CD14 Signaling Reciprocally Controls Collagen Deposition and Turnover to Regulate the Development of Lyme Arthritis

Bikash Sahay,* Anju Singh,*
Arumugam Gnanamani,[†] Rebeca L. Patsey,*
J. Edwin Blalock,[‡] and Timothy J. Sellati*

From the Center for Immunology and Microbial Disease,* Albany Medical College, Albany, New York; the Microbiology Division,[†] Central Leather Research Institute, Chennai, Tamil Nadu, India; and the Lung Health Center,[‡] Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

CD14 is a glycosylphosphatidylinositol-anchored protein expressed primarily on myeloid cells (eg, neutrophils, macrophages, and dendritic cells). CD14^{-/-} mice infected with *Borrelia burgdorferi*, the causative agent of Lyme disease, produce more proinflammatory cytokines and present with greater disease and bacterial burden in infected tissues. Recently, we uncovered a novel mechanism whereby CD14^{-/-} macrophages mount a hyperinflammatory response, resulting from their inability to be tolerized by *B. burgdorferi*. Paradoxically, CD14 deficiency is associated with greater bacterial burden despite the presence of highly activated neutrophils and macrophages and elevated levels of cytokines with potent antimicrobial activities. Killing and clearance of *Borrelia*, especially in the joints, depend on the recruitment of neutrophils. Neutrophils can migrate in response to chemotactic gradients established through the action of gelatinases (eg, matrix metalloproteinase 9), which degrade collagen components of the extracellular matrix to generate tripeptide fragments of proline-glycine-proline. Using a mouse model of Lyme arthritis, we demonstrate that CD14 deficiency leads to decreased activation of matrix metalloproteinase 9, reduced degradation of collagen, and diminished recruitment of neutrophils. This reduction in neutrophil numbers is associated with greater numbers of *Borrelia* in infected tissues. Variation in the efficiency of neutrophil-mediated clearance of *B. burgdorferi* may underlie differences in the severity of Lyme arthritis observed in the patient population

and suggests avenues for development of adjunctive therapy designed to augment host immunity. (Am J Pathol 2011, 178:724–734; DOI: 10.1016/j.ajpath.2010.10.025)

Lyme disease is caused by three related borrelial species found in North America (ie, *Borrelia burgdorferi*), Europe, and Asia (*Borrelia afzelii* and *Borrelia garinii*). In the United States the etiological agent is exclusively *B. burgdorferi*, which is transmitted by the deer tick *Ixodes scapularis*. *B. burgdorferi* is the most arthritogenic of the three species, with approximately 60% of untreated patients developing Lyme arthritis in response to the bacterium.¹ Recruitment of leukocytes [eg, neutrophils, macrophages, dendritic cells (DCs), and lymphocytes] to the site of infection is one of the more important mechanisms whereby spirochetes are cleared from tissues. Neutrophils are the principal early infiltrating cell type observed in the infected joints of humans,² a finding mirrored in a mouse model of Lyme arthritis.^{3–6} Depletion of neutrophils results in early onset of arthritis with a higher bacterial burden in murine Lyme borreliosis.⁴ Conversely, *B. burgdorferi* genetically engineered to express KC (the murine equivalent of human CXCL8), a neutrophil-recruiting chemokine, is rapidly cleared from mouse tissues because of a faster and continuous influx of neutrophils to the site of infection.⁶

The host response to invading pathogens often is typified by a local inflammatory reaction, which generates a gradient of CXCR1/2 ligands that promotes the transendothelial migration of neutrophils into sites of infection.^{7–10} Once there, neutrophils degranulate, thus releasing antimicrobial effectors, such as cathelicidin antimicrobial peptide,¹¹ CXC chemokines [eg, CXCL8/KC and macrophage inflammatory protein 2 (MIP-2)],¹² and extracellular matrix (ECM)–de-

Supported by U.S. Public Health Service grants AI075193 and AI054546 (T.J.S.) and a DBT-Overseas Associateship from the Department of Biotechnology, New Delhi (A.G.).

Accepted for publication October 21, 2010.

The authors did not disclose any relevant financial relationships.

Address reprint requests to Timothy J. Sellati, Center for Immunology and Microbial Disease, Albany Medical College, 47 New Scotland Ave., MC151, ME205B, Albany, NY 12208-3479. E-mail: sellatt@mail.amc.edu.

grading enzymes [eg, elastase and matrix metalloproteinase 9 (MMP-9)].¹² Of these molecules, elastase activity has been implicated in host defense against gram-negative bacteria¹³; however, a similar role for MMP-9 has not been firmly established. In fact, we recently reported a positive correlation between MMP-9 activity and susceptibility to infection with the gram-negative bacterium *Francisella tularensis*.¹⁴ Regardless of whether its role is protective or destructive, the ability of MMP-9 to orchestrate the continuous recruitment of neutrophils has been demonstrated using several infectious and noninfectious disease models.^{14–17}

MMPs belong to a multigenic family of proteinases that perform diverse functions, such as tissue remodeling during development, wound healing, establishment and maintenance of chemokine gradients, and cell migration.¹⁸ There are 26 known genes in humans encoding this class of metalloproteinases, which are grouped on the basis of substrate specificity (eg, gelatinases such as MMP-2 and MMP-9). Like many of the MMPs, MMP-9 is released from activated neutrophils and macrophages as a proenzyme whose N-terminus is cleaved by other proteinases (eg, plasmin) to generate an active gelatinase.^{19,20} The action of MMPs is regulated by a family of molecules known as tissue inhibitors of metalloproteinases (TIMPs), which act by binding either the latent or active form of the enzyme.¹⁸ Particularly relevant to the present study, elevated levels of MMP-9 have been observed in the erythema migrans lesions and cerebrospinal and synovial fluid of patients with Lyme disease.^{21–24} Once secreted, MMP-9 cleaves the ECM, thus facilitating cell migration through a normally dense fibrillar environment. In addition to fostering general leukocyte movement, degradation of collagen also liberates small tripeptide fragments such as proline-glycine-proline (PGP), which exhibit an affinity for the chemokine receptors CXCR1/2 as do the neutrophil chemoattractants CXCL8/KC and MIP-2.^{15,17,25} The PGP gradient thus formed serves to prolong recruitment of neutrophils even after the CXCL chemokine gradient wanes.^{15,17}

CD14 is a glycosylphosphatidylinositol-anchored protein expressed primarily on cells of myeloid origin, such as neutrophils, macrophages, and DCs. The current paradigm suggests CD14 acts as a coreceptor for Toll-like receptor 2 (TLR2) to facilitate elaboration of innate inflammatory responses to bacterial infection; however, infection of CD14^{−/−} mice with *B. burgdorferi*, which display an abundance of TLR2 agonists (ie, lipoproteins), leads to greater production of proinflammatory cytokines, more severe disease, and increased bacterial burden in a variety of tissues. Recently, we defined changes in *B. burgdorferi*-induced macrophage activation that occur in the absence of CD14 and their impact on negative regulation of NF- κ B signaling. Diminished negative regulation and thus an inability of macrophages to become tolerant to the stimulatory effects of *B. burgdorferi* underlie the “hyperactive” release of cytokines. However, one perplexing observation was impaired bacterial clearance in CD14^{−/−} mice despite increased levels of cytokines, such as tumor necrosis factor- α and interferon- γ , known for their antimicrobial activities. Herein, we reveal a unique and unanticipated role for CD14 in the activation of MMP-9 in *Borrelia*-infected joints that regulates the recruit-

ment of neutrophils, which are required for killing, and clearance of invading spirochetes.

Materials and Methods

Reagents

All buffers and reagents were prepared to minimize contamination with environmental lipopolysaccharide by using baked (180°C for 4 hours) and autoclaved glassware, disposable plasticware, and pyrogen-free H₂O.

Cultivation of *B. burgdorferi*

A low-passage, clonal isolate (AH130) of *B. burgdorferi* strain 297²⁶ was maintained at 23°C in Barbour-Stoenner-Kelley medium containing 6% normal rabbit serum from Pel-Freez Biologicals (Rogers, AR) and then temperature shifted to 37°C. A temperature-dependent increase in OspC expression was confirmed by silver staining of whole borrelial lysates separated by SDS polyacrylamide gel electrophoresis.

Mice and Infection Protocol

All mice of both sexes used in experiments were 4 to 8 weeks old. C3H/HeN (CD14^{+/+}) (Taconic, Germantown, NY), and FVB/N (MMP-9^{+/+}) mice (National Cancer Institute, Bethesda, MD) were housed in the Animal Resources Facility at Albany Medical College. Food and water were provided *ad libitum*, and all animal procedures conformed to Institutional Animal Care and Use Committee guidelines. CD14^{−/−} mice were generated as previously described²⁷ and subsequently backcrossed 10 generations onto a C3H/HeN background. FVB/N mice deficient in MMP-9 (MMP-9^{−/−}) were obtained from the Jackson Laboratory (Bar Harbor, ME).

Mice were infected via intradermal inoculation of 1 × 10⁵ spirochetes over the sternum. At 1-week intervals, tibiotarsal joint thickness was measured using digital calipers and bacterial burden in infected tissues was determined using isolated genomic DNA and quantitative real-time PCR (qPCR) as previously described. Total RNA also was isolated from infected tissues for qPCR as described below and elsewhere.

Myeloperoxidase Staining

Joints were excised at 3 and 6 weeks post infection (p.i.) and fixed in 10% neutral-buffered formalin. Tissues were processed using standard histologic methods to obtain 5- μ m-thick paraffin sections. Tissue sections were deparaffinized by incubation in 100% Xylene (3 minutes, 3×), 100% ethanol (3 minutes, 3×), 95% ethanol (3 minutes), 70% ethanol (3 minutes), 50% ethanol (3 minutes), and a final rinse in H₂O. Deparaffinized tissue sections were boiled in sodium citrate buffer (10 mmol/L sodium citrate, 0.05% Tween 20, pH 6.0) for 10 minutes in a microwave oven for antigen retrieval. Tissue sections were incubated in 3% H₂O₂ for 10 minutes to inactivate endogenous perox-

ides. Tissue sections then were washed with 0.01% Triton X-100 and blocked with 2.5% normal horse serum for 20 minutes. Myeloperoxidase (MPO) was detected using a rabbit polyclonal antibody (catalog no. ab15484, Abcam Inc., Cambridge, MA) and the ImmPRESS anti-rabbit immunoglobulin detection kit coupled with the NovaRED substrate kit (Vector Laboratories, Burlingame, CA).

B. burgdorferi–Macrophage Activation Assay

Bone marrow–derived macrophages were isolated and expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 20% L929 cell–conditioned medium as previously described. Macrophages were seeded into 6-well tissue culture–treated plates at a concentration of 1×10^6 cells/2 ml per well and allowed to adhere overnight. The following day, *B. burgdorferi* were enumerated and resuspended as described above. Macrophages were washed twice with serum-free DMEM to remove any traces of fetal bovine serum, and spirochetes (resuspended in DMEM + 4% autologous serum) were added at a multiplicity of infection of 10 and co-incubated for different intervals at 37°C with 5% CO₂. Cells incubated with DMEM + 4% autologous serum alone served as mock-infected controls.

Cytokine Analysis

Tissue homogenates were generated by suspending excised joints in 1 ml of PBS containing EDTA-free protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) and 1 mmol/L Bestatin (Sigma-Aldrich Co., St. Louis, MO) and subjecting them to physical disruption as previously described. Supernatants were clarified by microcentrifugation at $2000 \times g$ for 5 minutes. Cytokine levels were measured in joint tissue homogenates or culture supernatants using a Mouse Cytometric Bead Array Flex Set for the detection of KC. Analyses were performed on a FACSArray flow cytometer (BD Immunocytometry Systems, San Jose, CA), and data were acquired and analyzed using BD FACSArray software and FCAP Array software, version 1.0 (BD Immunocytometry Systems), respectively. Measurement of MIP-2 was accomplished using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instructions. Cytokine levels shown represent values normalized on the basis of milligrams of protein from which they were isolated.

Real-Time qPCR

Total RNA was isolated from macrophages using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions. RNA (0.5 μ g) was used for reverse transcription of cDNA using Superscript II (Invitrogen Corporation, Carlsbad, CA). cDNA was used to amplify the target genes listed in Table 1, and qPCR was performed as described elsewhere.

Table 1. Primer Sequences Used in qPCR

Gene		Primer sequence
<i>mmp-9</i>	F	5'-CGAAGCTTCGACACTGACAAGAAGT-3'
	R	5'-GCACGCTGGAATGATCTAAGC-3'
<i>col2a1</i>	F	5'-CAACACAATCCATTGCGAAC-3'
	R	5'-TCTGCCCAGTTCAGGTCTCT-4'
<i>col10a1</i>	F	5'-GCTTACCCAGCAGTAGGTGC-3'
	R	5'-TCTGTGAGCTCCATGATTGC-4'
18s RNA	F	5'-ATAGCTGTATATTAAAGTTG-3'
	R	5'-GTCCATTCCATTATTCC-4'

F, forward; R, reverse.

MMP-9 Zymography

Gelatinase activity was assayed by a modification of the method of Hibbs et al.²⁸ Briefly, 200 μ g of total protein from joint homogenates was incubated with 1 ml of sterile H₂O and 100 μ l of gelatin-agarose beads (Sigma-Aldrich) overnight at 4°C to enrich for gelatin-specific MMPs. Beads then were centrifuged, resuspended in nonreducing Laemmli sample buffer (lacking β_2 -mercaptoethanol), and incubated for 30 minutes at room temperature. Samples were not boiled before loading on a 7.5% nonreducing polyacrylamide gel containing SDS and 4 mg/ml of type A gelatin from porcine skin (Sigma-Aldrich). After electrophoresis, gels were washed and subjected to a denaturation/renaturation step to promote MMP activity without proteolytic cleavage of pro-MMP-9 or MMP-2. Gelatin activity was visualized by staining gels with 0.5% Coomassie blue and destaining with methanol/acetic acid; band intensities were quantified using a Fluorochem 8000 Imaging system (Cell Biosciences Inc., Santa Clara, CA) and the α FluorChem8900 software.

For densitometric analysis of the zymogram, the background intensity of each lane was subtracted from the band intensity of active MMP-9 and MMP-2 of the same lane. The corrected band intensity of active MMP-9 is presented as a ratio with the constitutively active MMP-2 from the same lane. These ratios then are plotted and compared with the ratio in uninfected joints, which was set to a value of 1.

Masson's Trichrome Staining

Tissue sections were deparaffinized as described above and incubated for 1 hour in preheated (60°C) Bouin's solution. Tissue sections were rinsed in distilled H₂O for 5 minutes and stained with hematoxylin for 10 minutes. Hematoxylin-stained sections were washed with H₂O and subjected to Masson's trichrome staining using the Accustain Trichrome Stain kit (Sigma-Aldrich) to assess the extent of collagen deposition and the structural integrity of fibrillar collagen in the joints.

Electron Spray Ionization–Liquid Chromatography–Mass Spectrometry/Mass Spectrometry

Total protein isolated from uninfected and infected joints from CD14^{+/+} and CD14^{−/−} mice were run on an MDS

Sciex API-4000 Q-Trap (Applied Biosystems, Foster City, CA) equipped with a Shimadzu high-performance liquid chromatogram (Shimadzu Corp., Kyoto, Japan) as previously described.¹⁴ This instrumentation has a limit of detection in the range of 10 to 20 pg/ml. The high-performance liquid chromatography is performed using a Develosil RP-Aqueous C30 (2.0 × 150 mm, 5 μm) column (Phenomenex, Torrance, CA) with a 20% to 100% gradient of H₂O containing 0.1% formic acid:acetonitrile. Gradients are run at 0.2 ml/min for 6 minutes followed by a 4-minute equilibration. Mass transitions for acetylated PGP were observed at 312-112 and 312-140. Mass transitions for nonacetylated PGP were observed at 270-70 and 270-116. Peak areas were integrated and quantified based on standard curves determined using known amounts of both forms of PGP under these conditions.

Statistical Analysis

Data were analyzed for statistical significance using a parametric two-way analysis of variance with a Tukey-Kramer multiple comparison if the data were found to fit a gaussian distribution (tested using the method by Kolmogorov and Smirnov). If not normally distributed, data were analyzed for statistical significance using a nonparametric two-way analysis of variance with the Kruskal-Wallis post *t*-test. When comparisons were made between wild-type and a CD14^{-/-} or MMP-9^{-/-} group, then a two-tailed paired *t*-test was used. Where bacterial burden between MMP-9^{+/+} and MMP-9^{-/-} infected mice were compared, a one-way analysis of variance with Student-Newman-Keuls multiple comparisons test was performed. An $\alpha = 0.05$ level was used to determine whether a significant difference existed between data from wild-type versus knockout and untreated versus treated groups.

Results

CD14 Regulates Neutrophil Recruitment into the Tibiotarsal Joints in Response to *B. burgdorferi* Infection

CD14^{-/-} C3H/HeN mice harbor 8 to 10 times more *Borrelia* in their joints than their wild-type counterparts. Neutrophils are the principal cell type that controls clearance of *Borrelia* in infected joints.³⁻⁵ Thus, to understand whether the higher spirochetal load in the joints of CD14^{-/-} mice is due to a lack of or diminished neutrophil recruitment, we used MPO staining to quantify the neutrophil content in tibiotarsal joints from infected animals. MPO, a lysosomal protein stored in azurophilic granules, serves as an ideal and specific marker for tracking the migration of neutrophils into inflamed tissues. The joints of CD14^{-/-} mice show a profound, nearly 50%, reduction in the number of recruited neutrophils both at 3 (data not shown) and 6 weeks p.i. (Figure 1, A and B).

Figure 1. Regulation of neutrophil influx by CD14. **A:** CD14^{+/+} and CD14^{-/-} C3H/HeN mice were infected intradermally with 1×10^5 *B. burgdorferi*. At 6 weeks p.i. neutrophil recruitment into joints was visualized by myeloperoxidase staining. Images are representative of transverse sections from a total of 10 mice of each genotype. **B:** Data represent a compilation of the total number of neutrophils enumerated in 4 transverse, nonconsecutive sections of CD14^{+/+} and CD14^{-/-} joints ($n = 5$ mice per group, for a total of 10 mice). Results are shown as means ± SEMs from two independent experiments. * $P < 0.0001$.

Differential Recruitment of Neutrophils Is Independent of CXCL Chemokine Levels

Neutrophils are recruited to the site of infection via a gradient of chemokines released by endothelial cells and resident macrophages and other cell types. We looked for evidence of two key neutrophil chemoattractants, KC and MIP-2, during infection of mice with *B. burgdorferi*. Total protein from the joints of CD14^{+/+} and CD14^{-/-} mice were evaluated for levels of these chemokines as determined by cytometric bead array or ELISA and were normalized to the total protein recovered from each joint. Although KC levels increased after infection, the presence or absence of CD14 had no significant influence on its production (Figure 2A). Although we failed to detect MIP-2 *in vivo*, *in vitro* stimulation of macrophages with *B. burgdorferi* elicited comparable levels of MIP-2 from either genotype and did so in a time-dependent fashion (Figure 2B).

CD14 Signaling Does Not Regulate MMPs or TIMPs at the Level of Transcription

Given the similar induction of chemokines observed and an appreciation of the role of MMPs and TIMPs in regulating the establishment of a gradient through turnover of

Figure 2. Influence of CD14 on *in vivo* and *in vitro* release of KC and MIP-2. **A:** CD14^{+/+} and CD14^{-/-} mice were infected intradermally with 1×10^5 *B. burgdorferi* and 3 weeks p.i. joints were excised and homogenized. Homogenates were assayed for KC by cytometric bead array and values were normalized to input protein from the joints. Results are shown as means $\pm$ SEMs from two independent experiments ($n = 6$ total). * $P < 0.05$, ** $P < 0.01$. **B:** CD14^{+/+} and CD14^{-/-} macrophages were co-incubated with *B. burgdorferi* (multiplicity of infection of 10) for the indicated length of time and the level of MIP-2 was measured by ELISA. Results are shown as means $\pm$ SEMs from two independent experiments ($n = 6$ total).

ECM, we evaluated whether CD14 deficiency influenced *B. burgdorferi*-induced transcription of genes encoding MMP family members (ie, MMP-2, -3, -8, -9, -10, -11, -12, and -13). Only levels of transcript for *mmp2*, *mmp3*, *mmp9*, *mmp10*, and *mmp13* were elevated after co-incubation of macrophages with *B. burgdorferi*. Although transcription above mock control for most genes did not exceed 5- to 10-fold, the induction of *mmp9* was approximately 100-fold (Figure 3). The absence of CD14 did not influence transcript levels of any of the genes analyzed (data not shown), including *mmp9*. To validate our *in vitro* finding, we assayed for *mmp9* transcripts in the joints of infected CD14^{+/+} and CD14^{-/-} mice. However, *in vivo* evidence of *B. burgdorferi*-induced *mmp9* transcription was lacking as has been reported elsewhere.²⁹ We also looked at members of the TIMP family by qPCR and observed decreased transcript levels of *timp2* and *timp3*, but not *timp1* or *timp4*, in *B. burgdorferi*-activated macrophages (data not shown). Again, there was no significant difference between the two genotypes.

CD14 Regulates the Activation of MMP-9 in *Borrelia*-Infected Mice

In addition to potential regulation at the transcriptional, posttranscriptional, and translational levels, the action of MMP-9 can be controlled at the posttranslational level as well. As such, we looked at the activation status of MMP-9 by gelatin zymography with the rationale that perhaps CD14 regulates the conversion of MMP-9 from its pro-form to its active form. As seen in Figure 4A, both inactive and active MMP-9 was detected in uninfected and infected mouse joints. The upper band corresponding to inactive MMP-9 was abundant in both genotypes and did not appear to increase significantly on infection of animals with *B. burgdorferi*. In contrast, active MMP-9 levels were elevated on infection and were higher in the joints of CD14^{+/+} than in CD14^{-/-} mice at 3 and 6 weeks p.i. (Figure 4, A and B).

Differences in CD14-Dependent MMP-9 Activity Are Reflected in the Extent of Collagen Turnover

Because active MMP-9 levels were lower in *B. burgdorferi*-infected CD14^{-/-} joints, one would predict the integrity of collagen to be greater than in wild-type mice. To test this hypothesis, collagen within *Borrelia*-infected joints was visualized with Masson's trichrome stain. In the absence of CD14, collagen structure within the joint space is far more intact (as evidenced by dark blue staining) than in the joints of CD14^{+/+} mice (Figure 5A). To quantify differences between the genotypes, blue color extraction using Adobe Photoshop was performed as previously described. Joints of CD14^{-/-} mice were found to have nearly twice as much collagen staining compared with their wild-type counterparts (Figure 5B). In addition, total protein preparations from isolated joints were assayed by mass spectrometry for the presence of acetylated PGP, which is generated by the degradation of collagen through the action of MMP-9. The levels of

Figure 3. Role of CD14 in transcriptional regulation of *mmp9*. Total RNA was isolated from CD14^{+/+} and CD14^{-/-} macrophages incubated with *B. burgdorferi* (multiplicity of infection of 10) for different periods and *mmp9* transcript levels were determined by qPCR. Results are shown as a fold change over mock-infected control macrophages and were normalized to 18S rRNA transcript levels. Data represent the means $\pm$ SEMs from three independent experiments. * $P < 0.01$, ** $P < 0.001$.

Figure 4. Effect of CD14 deficiency on posttranslational MMP-9 activation. **A:** CD14^{+/+} and CD14^{-/-} mice were infected intradermally with 1×10^5 *B. burgdorferi* and were sacrificed at 1, 3, and 6 weeks after infection. Joints were homogenized in PBS containing proteinase inhibitors. Gelatin zymography was performed using equal amounts of protein lysate. Data are presented as a negative image and are representative of two independent experiments. Inactive and active forms of MMP-9 and MMP-2 are denoted with an i or a, respectively. **B:** Active MMP-9 bands shown in panel A were subjected to densitometric analysis and quantified as detailed in the *Materials and Methods* section. Fetal bovine serum (FBS) served as a positive control for MMP-2 and MMP-9.

PGP in the joints of infected mice were significantly higher than those of uninfected control animals ($P = 0.00014$), and although not significantly different, there was a trend toward more PGP in the joints of CD14^{+/+} mice than in mice lacking CD14 (8.497 ± 0.6206 versus 7.215 ± 1.008). Finally, given prior evidence of dysregulation of gene transcription in the absence of CD14, we determined whether differences in collagen content between wild-type and CD14^{-/-} joints might also reflect greater transcription of collagen genes in the latter, where hyperinflammatory conditions exist. To explore this possibility, we measured the transcript levels of 10 different collagen genes at 3 weeks after inoculation. Two genes, *col2a1* and *col10a1*, were transcribed in the absence of CD14 to a greater, although not statistically significant, extent (data not shown).

B. burgdorferi-Infected Joints from MMP-9^{-/-} Mice Have Decreased Collagen Turnover

In the absence of CD14, we observed decreased MMP-9 activity and a corresponding maintenance of collagen integrity. To test directly whether differences in CD14-regulated MMP-9 activity were responsible, collagen integrity was evaluated in the *Borrelia*-infected joints of

FVB/N mice and their MMP-9-deficient (MMP-9^{-/-}) counterparts. In the absence of MMP-9, the integrity of collagen was maintained to a higher degree than in wild-type mice (Figure 6A) with approximately four fold greater collagen content in MMP-9^{-/-} mice than in those animals expressing MMP-9 (Figure 6B).

MMP-9 Deficiency Leads to Lower Recruitment of Neutrophils and Impaired Clearance of Borrelia from Joints

Finally, the extent of neutrophil recruitment into the joints of MMP-9^{+/+} and MMP-9^{-/-} mice was evaluated. As was observed in CD14^{-/-} joints (Figure 1), the joints of *B. burgdorferi*-infected mice lacking MMP-9 have significantly fewer neutrophils than MMP-9^{+/+} animals as evidenced by the amount of MPO staining at 3 (data not shown) and 6 weeks p.i. (Figure 7A). Data compiled from

Figure 5. Effect of CD14 deficiency on collagen turnover. **A:** CD14^{+/+} and CD14^{-/-} mice were infected intradermally with 1×10^5 *B. burgdorferi* and at 3 and 6 weeks p.i. processed joint sections were stained with Masson's trichrome to visualize collagen. Photographic images are shown at 4× and 100× (inset) magnification. Data are representative of two independent experiments ($n = 12$ total). **B:** Images of the joints at 6 week p.i. were imported to Adobe Photoshop, and their blue color was extracted and the area covered calculated as described elsewhere.

two independent experiments show a nearly 75% reduction in neutrophil recruitment in the absence of MMP-9 (Figure 7B). To validate our overall hypothesis that diminished MMP-9 activity results in lower neutrophil recruitment and impaired bacterial clearance from the joint, we evaluated bacterial burden in different organs of infected MMP-9^{+/+} and MMP-9^{-/-} mice. After infection of mice with 1×10^5 *B. burgdorferi*, genomic DNA was isolated from the heart, joint, ear, and bladder at 1, 3, and 6 weeks p.i., and qPCR was used to quantify bacterial burden. Both the genotypes of mouse were equally susceptible to infection and harbored similar numbers of bacteria in their organs at 1 and 3 weeks p.i. (Figure 8). However, by 6 weeks p.i. the bacterial burden was markedly greater in MMP-9^{-/-} joints than in those of wild-type mice ($P < 0.01$).

Discussion

During transmission of *B. burgdorferi* to the mammalian host, neutrophils are recruited to the tick feeding site. However, because of the neutrophil-inhibitory effects of *I.*

Figure 6. Effect of MMP-9 deficiency on collagen turnover. **A:** Joints from *Borrelia*-infected MMP-9^{+/+} and MMP-9^{-/-} mice were stained with Masson's trichrome to visualize collagen as detailed in the legend of Figure 5. Data are representative of two independent experiments ($n = 12$ total). **B:** Images of the joints at 6 week p.i. were processed as detailed in the legend of Figure 5.

Figure 7. Regulation of neutrophil influx by MMP-9. **A:** MMP-9^{+/+} and MMP-9^{-/-} mice were infected intradermally with 1×10^5 *B. burgdorferi*. At 6 weeks p.i., neutrophil recruitment into joints was visualized by myeloperoxidase staining. Images are representative of transverse sections from a total of 10 mice of each genotype. **B:** Data represent a compilation of the total number of neutrophils enumerated in four transverse, nonconsecutive sections of MMP-9^{+/+} and MMP-9^{-/-} joints ($n = 10$ total). Results are shown as means $\pm$ SEMs from two independent experiments. * $P < 0.0001$.

scapularis saliva, not all spirochetes are cleared from the site of inoculation.^{30,31} Subsequent dissemination to distal sites away from the effects of tick-salivary proteins allows for efficient phagocytosis and killing of *B. burgdorferi* by neutrophils. This scenario is supported by the finding that *Borrelia* infection of mice wherein neutrophils were depleted using anti-GR-1 antibody results in higher bacterial burden and early onset of arthritis.⁴ Similarly,

Figure 8. Effect of MMP-9 deficiency on clearance of *B. burgdorferi*. MMP-9^{+/+} and MMP-9^{-/-} mice were infected intradermally with 1×10^5 *B. burgdorferi*. At 1, 3, and 6 week p.i., DNA was isolated from infected tissues and the bacterial burden was determined using Taqman probes for *B. burgdorferi* *flaB* and murine *nidogen*, the latter serving as a normalization control. Results represent means $\pm$ SEMs from two independent experiments ($n = 10$ total) wherein samples were run in triplicate. * $P < 0.01$.

when *B. burgdorferi* genetically engineered to secrete KC were syringe inoculated into mice, the responding neutrophils effectively cleared spirochetes, thus short-circuiting the infectious process.⁶ This effect was reversed when *Borrelia*-expressed KC was neutralized by anti-KC antibodies.

CD14 facilitates interactions between bacterial products and the TLR signaling machinery, which promote innate immune responses. Nevertheless, its absence in mice and from monocytes/macrophages results in hyper-responsiveness to live *B. burgdorferi*, which leads to greater production of proinflammatory cytokines. One of the more paradoxical consequences of CD14 deficiency is that despite elevated levels of cytokines with potent antimicrobial activities, CD14^{-/-} mice harbor 10-fold more bacteria in their joints than do wild-type animals. This led us to examine whether CD14 signaling regulates the recruitment of neutrophils in a model of Lyme borreliosis. Traditionally, neutrophil recruitment is regulated through the establishment of a chemotactic gradient formed by the release of chemokines at the site of infection or noninfectious tissue injury. On recruitment, neutrophils release their granular contents into the extracellular milieu as a means to kill bacteria, remodel the ECM through the action of degradative enzymes, and recruit other leukocytes, such as macrophages. Neutrophils are short-lived cells; they die at the site of infection and then are cleared by the incoming macrophages and DCs. As the chemokine gradient wanes, the influx of neutrophils diminishes; however, in several inflammatory conditions where tissue injury is a dominant feature, neutrophils continue to enter inflamed tissues even in the absence of detectable levels of chemokines. Prolongation of neutrophil migration results from generation of collagen fragments released through turnover of the ECM.³²⁻³⁴ Weathington et al¹⁵ have demonstrated the neutrophil chemotactic potential of small tripeptide fragments of PGP generated by the action of MMP-9. The capacity of MMP-9 to regulate neutrophil recruitment and thus influence the intensity and duration of disease is well documented.³⁵ It is worth noting that correlation between MMP-9 activity and disease severity can vary and depends on whether the disease is infectious or noninfectious in origin.

For example, the severity of rheumatoid arthritis correlates positively with the level of active MMP-9 and the number of neutrophils recruited into the joint space.³⁶⁻³⁸ In infectious diseases, because neutrophils play a role in both tissue damage and clearance of pathogens, the balance between the two becomes delicate and essential to maintain. MMP-9 deficiency increases the survival of *F. tularensis*-infected mice by avoiding excessive neutrophil recruitment and greater tissue damage in the lung. However, at the other extreme, complete antibody-mediated depletion of neutrophils increases mortality after challenge of mice with a sublethal dose of *Francisella*.^{14,39} In a sepsis model, where MMP-9^{-/-} mice were challenged with *Escherichia coli*, animals showed severe impairment of neutrophil recruitment in late-phase disease and higher bacterial burden.⁴⁰ *Staphylococcus aureus*-triggered septic arthritis also is exacerbated by MMP-9 de-

ficiency wherein mice display a higher frequency and severity of arthritis and harbor increased numbers of bacteria in their joints.⁴¹ Similarly, as Lyme arthritis develops there is a tug-of-war between clearance of spirochetes (a necessary prelude to resolution of disease) and damage to tissues. Herein, we observed lower active MMP-9 in the joints of CD14^{-/-} mice, which was associated with greater maintenance of collagen integrity and a trend toward lower levels of PGP. Consistent with this observation were reduced numbers of neutrophils (especially at the height of the arthritic reaction) and impaired clearance of bacteria. MMP-9^{-/-} mice showed a similar phenotype, including maintenance of collagen integrity, fewer recruited neutrophils, and a higher spirochetal burden in joints at 6 weeks after initial infection. Recently, Heilpern et al⁴² evaluated the role of MMP-9 in dissemination of *B. burgdorferi* and reported no differences in the rate and/or pattern of spirochetal dissemination, bacterial burden, cytokine/chemokine production, or the extent and/or composition of cellular infiltrates (including neutrophils). However, they found a lesser degree of joint swelling in the absence of MMP-9, the opposite of what we observed (data not shown). Discordance among the findings of Heilpern et al, the results presented herein, and our current understanding of the role of MMP-9 in mediating neutrophil recruitment may be due to differences in the genetic background of mice used (C3H/HeN in the study by Heilpern et al and FVB/N in the present study). However, a potentially more satisfying and likely explanation is that experiments in the study by Heilpern et al were terminated at 3.5 weeks p.i., whereas, in the present study, significant differences in the severity of Lyme arthritis were more noticeable at 6 weeks. Looking for the potential impact of MMP-9 deficiency during late-phase disease is necessary because similar levels of CXCL neutrophil chemokines were observed in wild-type and knockout mice during the height of the arthritic response (2 to 4 weeks). However, later in the disease process (ie, 6 weeks in the present study), once overall chemokine levels wane, differences in the influence of MMP-9-generated chemotactic peptides were unmasked.

In response to *B. burgdorferi*, *in vitro* and *in vivo* transcription and expression of MMP-9, but not its post-translational activation, occurred in a CD14-independent manner. In contrast, Zhao et al²⁴ demonstrated a requirement for CD14 in both the transcription and expression of MMP-9 in human macrophages and fibroblasts. Left unanswered because of differences in experimental design is whether CD14 has a species-specific role in posttranslational activation of MMP-9. One critical distinction between the two studies is that Zhao et al relied entirely on *in vitro* experimentation with immortalized human monocytic and fibroblastic cell lines (ie, U937 and ATCC SCRC-1042.2, respectively), whereas, in the present study, the role of CD14 in regulation of MMP-9 at the transcriptional, translational, and posttranslational levels was evaluated during natural infection, allowing for more complex inter-

actions among the pathogen, a variety of host cell types, and the ECM.

Finally, a phenomenon indicative of fibrosis reported herein was greater collagen staining in the joints of infected CD14^{-/-} versus CD14^{+/+} mice. Inflammation and fibrosis are two interrelated processes with intertwined signaling events, especially when elicited by infectious agents. As part of the tissue repair process, neutrophils and macrophages coordinate their efforts to clear residual bacterial components and effect removal of apoptotic and necrotic cellular debris from the site of infection. Chemokines, cytokines, and growth factors released by these phagocytes recruit lymphocytes to and activate fibroblasts within the inflammatory focus.^{43,44} In addition, the differentiation of macrophages into fibroblast-like cells called myofibroblasts occurs within these foci. Fibroblasts and myofibroblasts participate in the reconstruction of ECM by virtue of their ability to deposit collagen and secrete MMPs. Tissue repair, a highly regulated process, is subject to dysregulation in the prolonged presence of bacteria and cytokines.^{45–47} Perpetual TLR stimulation at sites of infection or noninfectious injury can amplify profibrotic signaling and engender fibrotic disorders.^{45–47} In renal injury and cystic fibrosis, excessive TLR2 signaling induces several fibrotic genes.^{45–48} The absence of CD14 on macrophages results in a 50-fold increase in *B. burgdorferi*-stimulated transcription of *tlr2* and protracted surface expression of protein, which, in turn, is partly responsible for greater production of proinflammatory cytokines. The ability of macrophages to trigger apoptosis in myofibroblasts also may depend on CD14 expression. Thus, in its absence, extended survival of myofibroblasts and their accumulation within joints could contribute to greater collagen deposition in CD14^{-/-} mice. Alternatively, the inability of spirochetes to induce inducible nitric oxide synthase in CD14^{-/-} macrophages may be a factor because the absence of inducible nitric oxide synthase activity has been associated with greater transcription of collagen genes and higher collagen deposition.⁴⁹ Although not significant, a trend toward higher transcription of collagen genes was observed in CD14^{-/-} joints compared with those of wild-type mice. Considering the potential implications of greater deposition and/or diminished turnover of ECM, it is important to note that *B. burgdorferi* express decorin-binding proteins A and B, especially when they reside in the skin and joints where collagen content is high.⁵⁰ Surface expression of decorin-binding proteins A and B anchors spirochetes to collagen^{50,51} and may facilitate their persistence in mouse and human joints. The ability of spirochetes to embed themselves in dense collagen bundles may offer protection from both antibodies and antibiotics.^{52–55} Putting all this information together, we can speculate that CD14 not only helps in the activation of MMP-9 necessary for degradation of existing collagen but also may limit the continued production of collagen. Control of these fibrotic processes by CD14 not only leads to higher neutrophil recruitment and clearance of spirochetes but also deprives *B. burgdorferi* of “hide-outs” within joints.

This study describes a previously unappreciated function for CD14 in bacterial clearance by regulating the extravasation of neutrophils into the joints of *B. burgdorferi*-infected mice. Although CD14 deficiency leaves unaltered the production of CXCL neutrophil chemoattractants and transcription of *mmp-9* both *in vitro* and *in vivo*, a significant decrease in the pool of active MMP-9 occurs. The cascade effect of this reduction in MMP-9 activity was diminished turnover of ECM and generation of PGP, limited recruitment of neutrophils into the joint space, and impaired clearance of spirochetes. These findings raise the intriguing question of whether individual variation in the efficiency of neutrophil-mediated clearance of *B. burgdorferi* might underlie differences in the severity and/or duration of Lyme arthritis observed in the patient population. If such differences are responsible for the range of clinical presentation observed, it suggests avenues for the development of adjunctive therapy designed to augment host immunity, perhaps through small molecule-mediated enhancement of neutrophil recruitment.

Acknowledgments

We thank Drs. Michael DiPersio (Albany Medical College, Albany, NY) and Martha Furie (Stony Brook University, Stony Brook, NY) for critical review of the manuscript. We are indebted to Dr. Paul Feustal for discussions on biostatistics and Yili Lin (Director, Immunology Core at Albany Medical College) for providing technical support for flow cytometry-based studies.

References

1. Steere AC, Glickstein L: Elucidation of Lyme arthritis. *Nat Rev Immunol* 2004, 4:143–152
2. Duray PH, Steere AC: Clinical pathologic correlations of Lyme disease by stage. *Ann N Y Acad Sci* 1988, 539:65–79
3. Brown CR, Blaho VA, Loiacono CM: Susceptibility to experimental Lyme arthritis correlates with KC and monocyte chemoattractant protein-1 production in joints and requires neutrophil recruitment via CXCR2. *J Immunol* 2003, 171:893–901
4. Brown CR, Blaho VA, Loiacono CM: Treatment of mice with the neutrophil-depleting antibody RB6-8C5 results in early development of experimental Lyme arthritis via the recruitment of Gr-1-polymorphonuclear leukocyte-like cells. *Infect Immun* 2004, 72:4956–4965
5. Montgomery RR, Booth CJ, Wang X, Blaho VA, Malawista SE, Brown CR: Recruitment of macrophages and polymorphonuclear leukocytes in Lyme carditis. *Infect Immun* 2007, 75:613–620
6. Xu Q, Seemanapalli SV, Reif KE, Brown CR, Liang FT: Increasing the recruitment of neutrophils to the site of infection dramatically attenuates *Borrelia burgdorferi* infectivity. *J Immunol* 2007, 178:5109–5115
7. Sellati TJ, Burns MJ, Ficazzola MA, Furie MB: *Borrelia burgdorferi* upregulates expression of adhesion molecules on endothelial cells and promotes transendothelial migration of neutrophils *in vitro*. *Infect Immun* 1995, 63:4439–4447
8. Wooten RM, Modur VR, McIntyre TM, Weis JJ: *Borrelia burgdorferi* outer membrane protein A induces nuclear translocation of nuclear factor-kappa B and inflammatory activation in human endothelial cells. *J Immunol* 1996, 157:4584–4590
9. Sellati TJ, Abrescia LD, Radolf JD, Furie MB: Outer surface lipoproteins of *Borrelia burgdorferi* activate vascular endothelium *in vitro*. *Infect Immun* 1996, 64:3180–3187

10. Burns MJ, Sellati TJ, Teng EI, Furie MB: Production of interleukin-8 (IL-8) by cultured endothelial cells in response to *Borrelia burgdorferi* occurs independently of secreted [corrected] IL-1 and tumor necrosis factor alpha and is required for subsequent transendothelial migration of neutrophils. *Infect Immun* 1997, 65:1217–1222
11. Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, Gennaro R: Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem* 1997, 272:13088–13093
12. Nathan C: Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 2006, 6:173–182
13. Belaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, Shapiro SD: Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med* 1998, 4:615–618
14. Malik M, Bakshi CS, McCabe K, Catlett SV, Shah A, Singh R, Jackson PL, Gaggari A, Metzger DW, Melendez JA, Blalock JE, Sellati TJ: Matrix metalloproteinase 9 activity enhances host susceptibility to pulmonary infection with type A and B strains of *Francisella tularensis*. *J Immunol* 2007, 178:1013–1020
15. Weathington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, Folkerts G, Nijkamp FP, Blalock JE: A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* 2006, 12:317–323
16. Gaggari A, Li Y, Weathington N, Winkler M, Kong M, Jackson P, Blalock JE, Clancy JP: Matrix metalloproteinase-9 dysregulation in lower airway secretions of cystic fibrosis patients. *Am J Physiol Lung Cell Mol Physiol* 2007, 293:L96–L104
17. Gaggari A, Jackson PL, Noerager BD, O'Reilly PJ, McQuaid DB, Rowe SM, Clancy JP, Blalock JE: A novel proteolytic cascade generates an extracellular matrix-derived chemoattractant in chronic neutrophilic inflammation. *J Immunol* 2008, 180:5662–5669
18. Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003, 92:827–839
19. Wang F-q, So J, Reierstad S, Fishman DA: Matrilysin (MMP-7) promotes invasion of ovarian cancer cells by activation of progelatinase. *Int J Cancer* 2005, 114:19–31
20. Gong Y, Hart E, Shchurin A, Hoover-Plow J: Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. *J Clin Invest* 2008, 118:3012–3024
21. Hu LT, Eskildsen MA, Masgala C, Steere AC, Arner EC, Pratta MA, Grodzinsky AJ, Loening A, Perides G: Host metalloproteinases in Lyme arthritis. *Arthritis Rheum* 2001, 44:1401–1410
22. Kirchner A, Koedel U, Fingerle V, Paul R, Wilske B, Pfister HW: Upregulation of matrix metalloproteinase-9 in the cerebrospinal fluid of patients with acute Lyme neuroborreliosis. *J Neurol Neurosurg Psychiatry* 2000, 68:368–371
23. Lin B, Kidder JM, Noring R, Steere AC, Klempner MS, Hu LT: Differences in synovial fluid levels of matrix metalloproteinases suggest separate mechanisms of pathogenesis in Lyme arthritis before and after antibiotic treatment. *J Infect Dis* 2001, 184:174–180
24. Zhao Z, Chang H, Trevino RP, Whren K, Bhawan J, Klempner MS: Selective up-regulation of matrix metalloproteinase-9 expression in human erythema migrans skin lesions of acute Lyme disease. *J Infect Dis* 2003, 188:1098–1104
25. Blalock JE: The immune system as the sixth sense. *J Intern Med* 2005, 257:126–138
26. Hubner A, Yang X, Nolen DM, Popova TG, Cabello FC, Norgard MV: Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc Natl Acad Sci U S A* 2001, 98:12724–12729
27. Haziot A, Ferrero E, Kontgen F, Hijiya N, Yamamoto S, Silver J, Stewart CL, Goyert SM: Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 1996, 4:407–414
28. Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL: Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J Biol Chem* 1985, 260:2493–2500
29. Behera AK, Hildebrand E, Scagliotti J, Steere AC, Hu LT: Induction of host matrix metalloproteinases by *Borrelia burgdorferi* differs in human and murine Lyme arthritis. *Infect Immun* 2005, 73:126–134
30. Guo X, Booth CJ, Paley MA, Wang X, DePonte K, Fikrig E, Narasimhan S, Montgomery RR: Inhibition of neutrophil function by two tick salivary proteins. *Infect Immun* 2009, 77:2320–2329
31. Montgomery RR, Lusitani D, de Boisfleury CA, Malawista SE: Tick saliva reduces adherence and area of human neutrophils. *Infect Immun* 2004, 72:2989–2994
32. Chang C, Houck JC: Demonstration of the chemotactic properties of collagen. *Proc Soc Exp Biol Med* 1970, 134:22–26
33. Pfister RR, Haddox JL, Lam KW, Lank KM: Preliminary characterization of a polymorphonuclear leukocyte stimulant isolated from alkali-treated collagen. *Invest Ophthalmol Vis Sci* 1988, 29:955–962
34. Pfister RR, Haddox JL, Sommers CI: Alkali-degraded cornea generates a low molecular weight chemoattractant for polymorphonuclear leukocytes. *Invest Ophthalmol Vis Sci* 1993, 34:2297–2304
35. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L: Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 2000, 80:617–653
36. Cascão R, Rosário HS, Souto-Carneiro MM, Fonseca JE: Neutrophils in rheumatoid arthritis: more than simple final effectors. *Autoimmun Rev* 2010, 9:531–535
37. Burrage PS, Mix KS, Brinckerhoff CE: Matrix metalloproteinases: role in arthritis. *Front Biosci* 2006, 11:529–543
38. Chang YH, Lin IL, Tsay GJ, Yang SC, Yang TP, Ho KT, Hsu TC, Shiao MY: Elevated circulatory MMP-2 and MMP-9 levels and activities in patients with rheumatoid arthritis and systemic lupus erythematosus. *Clin Biochem* 2008, 41:955–959
39. Sjöstedt A, Conlan JW, North RJ: Neutrophils are critical for host defense against primary infection with the facultative intracellular bacterium *Francisella tularensis* in mice and participate in defense against reinfection. *Infect Immun* 1994, 62:2779–2783
40. Renckens R, Roelofs JJTH, Florquin S, de Vos AF, Lijnen HR, van'T Veer C, van der Poll T: Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. *J Immunol* 2006, 176:3735–3741
41. Calander A-M, Starckx S, Opdenakker G, Bergin P, Quidling-Järbrink M, Tarkowski A: Matrix metalloproteinase-9 (gelatinase B) deficiency leads to increased severity of *Staphylococcus aureus*-triggered septic arthritis. *Microb Infect* 2006, 8:1434–1439
42. Heilpern AJ, Wertheim W, He J, Perides G, Bronson RT, Hu LT: Matrix metalloproteinase 9 plays a key role in Lyme arthritis but not in dissemination of *Borrelia burgdorferi*. *Infect Immun* 2009, 77:2643–2649
43. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat M-L, Gabbiani G: The myofibroblast: one function, multiple origins. *Am J Pathol* 2007, 170:1807–1816
44. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, Ikeda Y: Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leukoc Biol* 2003, 74:833–845
45. Greene CM, Branagan P, McElvaney NG: Toll-like receptors as therapeutic targets in cystic fibrosis. *Exp Opin Ther Targets* 2008, 12:1481–1495
46. Muir A, Soong G, Sokol S, Reddy B, Gomez MI, van Heeckeren A, Prince A: Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* 2004, 30:777–783
47. Shuto T, Furuta T, Oba M, Xu H, Li JD, Cheung J, Gruenert DC, Uehara A, Suico MA, Okiyonedo T, Kai H: Promoter hypomethylation of Toll-like receptor-2 gene is associated with increased proinflammatory response toward bacterial peptidoglycan in cystic fibrosis bronchial epithelial cells. *FASEB J* 2006, 20:782–784
48. Leemans JC, Butter LM, Pulsken WPC, Teske GJD, Claessen N, van der Poll T, Florquin S: The role of Toll-like receptor 2 in inflammation and fibrosis during progressive renal injury. *PLoS One* 2009, 4:e5704
49. Niu X-L, Yang X, Hoshiai K, Tanaka K, Sawamura S, Koga Y, Nakazawa H: Inducible nitric oxide synthase deficiency does not affect the susceptibility of mice to atherosclerosis but increases collagen content in lesions. *Circulation* 2001, 103:1115–1120
50. Liang FT, Brown EL, Wang T, Iozzo RV, Fikrig E: Protective niche for *Borrelia burgdorferi* to evade humoral immunity. *Am J Pathol* 2004, 165:977–985
51. Xu Q, Seemanapalli SV, McShan K, Liang FT: Increasing the interaction of *Borrelia burgdorferi* with decorin significantly reduces the 50 percent infectious dose and severely impairs dissemination. *Infect Immun* 2007, 75:4272–4281

52. Priem S, Burmester GR, Kamradt T, Wolbart K, Rittig MG, Krause A: Detection of *Borrelia burgdorferi* by polymerase chain reaction in synovial membrane, but not in synovial fluid from patients with persisting Lyme arthritis after antibiotic therapy. *Ann Rheum Dis* 1998, 57:118–121
53. Hodzic E, Feng S, Holden K, Freet KJ, Barthold SW: Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. *Antimicrob Agents Chemother* 2008, 52:1728–1736
54. Haupl T, Hahn G, Rittig M, Krause A, Schoerner C, Schonherr U, Kalden JR, Burmester GR: Persistence of *Borrelia burgdorferi* in ligamentous tissue from a patient with chronic Lyme borreliosis. *Arthritis Rheum* 1993, 36:1621–1626
55. Barthold SW, Hodzic E, Tunev S, Feng S: Antibody-mediated disease remission in the mouse model of Lyme borreliosis. *Infect Immun* 2006, 74:4817–4825