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Bb2Bb3 Regulation of Murine Lyme Arthritis Is Distinct from *Ncf1* and Independent of the Phagocyte Nicotinamide Adenine Dinucleotide Phosphate Oxidase

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Several quantitative trait loci regulating murine Lyme arthritis severity have been mapped, including a highly significant linkage found on chromosome 5, termed *Bb2Bb3*. Within this region, the *Ncf1* gene of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has recently been identified as a major regulator of arthritis severity in rodent models of rheumatoid arthritis, an effect attributed to protective properties of reactive oxygen species. To assess the role of *Ncf1* in Lyme arthritis, we introgressed *Bb2Bb3* from severely arthritic C3H/He mice onto mildly arthritic C57BL/6 mice. This increased Lyme arthritis severity, whereas the reciprocal transfer conferred protection from disease. A single nucleotide polymorphism was identified in the *Ncf1* gene that did not influence the protein sequence or expression of *Ncf1*. Although polymorphonuclear leukocytes from C57BL/6 mice generated a greater oxidative burst than polymorphonuclear leukocytes from C3H/He mice, studies with the *Bb2Bb3* congenic mice demonstrated this difference was not linked to *Ncf1* alleles. Furthermore, Lyme arthritis severity was not altered in mice lacking either the *Ncf1* or *Gp91phox* subunits of the NADPH oxidase complex. Together, these results argue that *Ncf1* is not a candi-

date gene for regulation of Lyme arthritis and reveal Lyme arthritis to be independent of NADPH oxidase activity, distinguishing it from other models of rheumatoid arthritis. (Am J Pathol 2005, 167:775–785)

Lyme disease is caused by the tick-borne spirochete *Borrelia burgdorferi* and can involve multiple organs including the joints, heart, skin, and nervous system.¹ Lyme arthritis is a consequence of bacterial invasion of joint tissue and results from localized inflammation triggered by persistent infection.^{2,3} Arthritis develops weeks to months after transmission from an infected tick and is characterized by edema, inflammatory cell infiltration, and hyperproliferation of synovium.^{1,4,5} In most individuals, arthritis will resolve after clearance of the bacteria, however, a subset of individuals with the most severe inflammatory arthritis have been documented to develop treatment-resistant arthritis that does not respond to antibiotic therapy.^{6,7} It is suspected that in these individuals progression to an autoimmune-mediated arthritis more similar to rheumatoid arthritis has occurred. Susceptibility to treatment-resistant arthritis is associated with HLA-DR1*0401, a major histocompatibility complex (MHC) class II allele also associated with rheumatoid arthritis.^{6,8,9} These studies suggest that there are two distinct stages of Lyme arthritis; an acute inflammatory arthritis dependent on bacterial invasion and persistence in joint tissues and a chronic, treatment-resistant arthritis that may represent a transition to a rheumatoid-like disease.

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Although mice do not develop treatment-resistant, autoimmune-linked Lyme arthritis they do provide an excellent model for the acute arthritis more commonly seen in humans. Barthold and colleagues⁴ initially described differences in arthritis severity in inbred strains of mice, and we have used this information as the foundation of a genetic mapping approach to identify quantitative trait loci (QTL) that regulate arthritis severity. C3H/He mice, including both the C3H/HeN and C3H/HeJ substrains, develop severe arthritis when infected with *B. burgdorferi* whereas C57BL/6 mice develop mild to moderate disease.^{4,10,11} In BALB/c mice arthritis severity is dependent on the infectious dose with progressively more severe arthritis developing at increasing inoculum dose.¹⁰ Several different intercross populations from these strains were infected with *B. burgdorferi* and microsatellite linkage analysis was performed to identify QTL linked to arthritis severity. The regulation of Lyme arthritis was revealed to be multigenic, with QTL identified on five different chromosomes: 1, 4, 5, 11, and 12.^{11,12} Of particular interest was a region spanning 44 to 80 cM on chromosome 5 that was identified in four different intercross populations derived from crosses between C3H/He mice and both C57BL/6 or BALB/c mice.¹² This region, termed *Bb2Bb3*, possesses at least four distinct QTL regulating various aspects of arthritis severity, including ankle swelling, histopathologically assessed arthritis severity, and tendon sheath thickness. Logarithm of odds (LOD) scores for linkage to *Bb2Bb3* ranged from 3.5 to 10.2, with the strongest linkage to arthritis severity mapping to 72 cM.¹²

Several studies have implicated the multisubunit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of phagocytic cells as a regulator of rodent models of rheumatoid arthritis. The multisubunit NADPH oxidase complex, which includes Ncf1 (p47phox), Ncf2 (p67phox), Cyba (p22phox), Cybb (gp91phox), Ncf4 (p40phox), and Rac1/2 (p21-Rac1/2), is required for the production of superoxide by phagocytic cells and is an important aspect of the host defense against certain pathogens.^{13–17} Neutrophil cytosolic factor 1, *Ncf1*, was found to be polymorphic in rats, and alleles encoding structurally distinct proteins were found to regulate the magnitude of the oxidative burst produced by stimulated polymorphonuclear leukocytes (PMNs).¹⁸ Rat strains with greater NADPH oxidase activity were protected from collagen, pristane, oil, and hexadecane arthritis, whereas strains with less active NADPH oxidase activity developed severe arthritis. NADPH oxidase activity was also found to regulate arthritis severity in zymosan- and immune complex-induced models of rheumatoid arthritis in mice. Mice deficient in the Ncf1 or Gp91phox subunits of the NADPH oxidase complex developed significantly more severe arthritis than wild-type controls.¹⁹ In addition a splicing mutation of *Ncf1*, resulting in low levels of truncated Ncf1 protein and a defective oxidative burst response, led to severe collagen-induced chronic arthritis in B10.Q mice.²⁰ These studies indicate that higher levels of reactive oxygen intermediates can modulate arthritis severity in several animal models.

Certain features of both Lyme arthritis and rheumatoid arthritis suggest a common mechanism could be contributing to joint lesions in both diseases. These features include similar histopathological findings in Lyme arthritis and early rheumatoid arthritis as well as the characteristic involvement and requirement for PMNs in both processes.^{4,21–23} In addition, acute Lyme arthritis can set the stage for autoimmune-mediated arthritis in a small group of susceptible individuals.^{6,8,9} The recent discovery that polymorphisms in *Ncf1* gene regulate the severity of rat models of rheumatoid arthritis by regulating the activity of the NADPH oxidase complex suggested that the magnitude of the phagocyte oxidative burst might also regulate Lyme arthritis severity. This possibility was made even more compelling by the positioning of mouse *Ncf1* at 74 cM on chromosome 5, within the *Bb2Bb3* locus and near a linkage carrying a LOD score of 10.2.

This study addresses the possibility that polymorphisms in *Ncf1* could regulate Lyme arthritis severity. Congenic mouse lines were developed in which the *Bb2Bb3* locus of C3H/He mice was introgressed onto C57BL/6 mice, and the same region of C57BL/6 mice was introgressed onto C3H/He mice. Introgression of *Bb2Bb3* onto either genetic background transferred the Lyme arthritis severity phenotype, confirming the presence of a strong regulatory locus in this region. To determine whether alleles of *Ncf1* contributed to arthritis severity phenotypes, the *Ncf1* gene from each strain in our studies was sequenced and transcript and protein levels were determined. A single nucleotide polymorphism (SNP) was identified in the *Ncf1* gene; however, it did not influence transcription or translation of Ncf1. PMNs from C57BL/6 mice displayed a stronger oxidative burst than PMNs from C3H/He mice; however, this phenotype was not transferred in *Bb2Bb3* congenic mice indicating it could not be due to allelic differences in *Ncf1*, or other linked genes in this region. Further evidence that NADPH oxidase activity did not regulate Lyme arthritis severity came from infection of C57BL/6 mice deficient in either the Ncf1 or Gp91phox subunits of the NADPH oxidase complex: arthritis remained mild in the oxidase-deficient mice. These results indicate a fundamental difference between Lyme arthritis and several rodent models for rheumatoid arthritis including adjuvant-, zymosan-, oil-, and collagen-induced arthritis.

Materials and Methods

Mice

C3H/HeNCr, C57BL/6NCr, and BALB/cAnNCr mice were obtained from the National Cancer Institute (Bethesda, MD); B6.129S2-*Ncf1^{tm1Shl}* (*Ncf1*^{−/−}) mice were obtained from Taconic Farms (Germantown, NY); and B6.129S6-*Cybb^{tm1Din}*/J (*Gp91phox*^{−/−}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Ncf1*^{−/−} mice were then maintained as homozygous breeding pairs and administered sulfamethoxazole trimethoprim (J.A. Webster, Sterling, MA) antibiotics in water. Antibiotics were removed 3 days before infection. Mice were housed

in the Animal Resource Center at the University of Utah Health Science Center (Salt Lake City, UT), according to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

Generation of Congenic Mouse Lines

C3.B6-*Bb2Bb3*N7F2 and B6.C3-*Bb2Bb3*N7F3 congenic mouse lines were generated by introgressing the region of chromosome 5 spanning *Bb2* and *Bb3* (D5Mit355 to D5Mit292) from C3H/He or C57BL/6 mice onto the reciprocal parental strain, C57BL/6NCr and C3H/HeNCr, respectively, by backcrossing seven generations, beginning with BXH (C57BL/6J × C3H/HeJ) recombinant inbred lines 4 or 6 from The Jackson Laboratory. Microsatellite analysis with appropriate primers, obtained from Research Genetics (Carlsbad, CA), was used to identify mice carrying the introgressed *Bb2Bb3* region and to eliminate known contaminating regions from the BXH mice, as determined from the JAX website [Mouse Genome Database (MGD), Mouse Genome Informatics web site: Recombinant Inbred Strain Distribution Patterns, The Jackson Laboratory, Bar Harbor, ME (URL: <http://www.informatics.jax.org/searches/riset.cgi?sort=cM&format=html&set=BXH&chromosome=>)]. Congenic mouse lines homozygous for the introgressed *Bb2Bb3* were generated by filial mating.

B. burgdorferi Culture and Infection

Spirochetes were cultured in Barbour-Stoenner-Kelly II (BSK II) medium containing 6% rabbit serum (Sigma, St. Louis, MO) for 4 days before infection. Mice were infected by intradermal injection at 6 to 7 weeks of age with 2×10^3 (unless otherwise indicated) passage 5 spirochetes of the N40 isolate of *B. burgdorferi* (provided by S. Barthold, University of California, Davis, CA). Control animals were intradermally injected with sterile BSK II containing 6% rabbit serum.

Assessment of Arthritis Severity

Rear ankle joints were measured at the time of infection and at 4 weeks after infection using a metric caliper. Measurements were taken of the thickest anteroposterior portion of the ankle with the joint extended. Data are reported as the change in ankle swelling. Histological assessment of arthritis severity was performed on the most swollen ankle joint. Joints were fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin, sectioned at 5 μ m thickness, mounted on glass slides, and stained with hematoxylin and eosin. The joint sections were evaluated in a blinded manner and scored for severity of injury on a subjective scale ranging from 0 to 5. A score of 0 indicated no lesions; 1 indicated minimal; 2, mild; 3, moderate; 4, marked; and 5, severe lesions. The overall lesion score represented a combined assessment of neutrophil infiltration, mononuclear cell infiltration, tendon sheath thickness, and reactive/repairative responses.

Isolation of DNA from Mouse Tissues

DNA was isolated from ankle, heart, and ear tissues collected at the time of sacrifice as previously described.²⁴ Briefly, tissues were incubated in 0.1% collagenase A (Roche, Indianapolis, IN) overnight at 37°C. Samples were then incubated in an equal volume of 0.2 mg/ml Proteinase K (Life Technologies, Inc., Rockville, MD) overnight at 55°C. DNA was recovered by phenol chloroform extraction, treated with RNase, and ethanol precipitated.

Quantification of *B. burgdorferi* DNA in Host Tissues

B. burgdorferi levels in host tissues was assessed using quantitative polymerase chain reaction (PCR) on the LightCycler (Roche).²⁵ Copy number of *B. burgdorferi* *RecA* and the single copy mouse gene *Nidogen* were calculated using the crossing point determined by Roche LightCycler Software. *RecA* values were then normalized to *Nidogen* copy number. The oligonucleotide primers used for detection of *B. burgdorferi* *RecA* are ntm17.F (5'-GTGGATCTATTGTATTAGATGAGGCTCTCG-3') and ntm17.R (5'-GCCAAAGTTCTGCAACATTAACACCTAAG-3'). The oligonucleotide primers used for detection of mouse *Nidogen* are nido.F (5'-CCAGCCACAGAATACCATCC-3') and nido.R (5'-GGACATACTCTGCTGCCATC-3').

Recruitment of Peritoneal Neutrophils

Mice were injected intraperitoneally with 0.5 ml of sterile 3% thioglycollate. After 4 hours mice were sacrificed and cells were recovered by peritoneal lavage [using phosphate-buffered saline (PBS) for isolation of RNA or Hank's balanced salt solution (HBSS) with 5% fetal calf serum and 10 mmol/L HEPES for the oxidative burst assay]. Red blood cells were eliminated by ammonium chloride/potassium lysis. Cytospin preparations from 2×10^5 cells were stained with the modified Wright Giemsa stain Hema 3 (Fisher Diagnostics, Middletown, VA) to determine the percent neutrophils.

Real-Time Quantitative Reverse Transcriptase (RT)-PCR

RNA was isolated from peritoneal neutrophils using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR on 5 μ g of total RNA was performed using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). *Ncf1* transcript levels were quantified using primers designed to detect a 5' and a 3' region of *Ncf1*, which were then normalized to β -actin. Quantitative PCR was performed using the LightCycler (Roche). The oligonucleotide primers used to detect 5' *Ncf1* are ncf1.b (5'-TCCAGGAGCTTATGAATGACC-3') and ncf1.c (5'-AGCCCCCTTGACAGTCCCGAC-3'), 3' *Ncf1* are ncf1.9.F

(5'-ATTTCCTCCATCCATGTATC-3') and *ncf1.10.R* (5'-CCTTTGTGCCATCCGTGC-3'), and *β -actin* are bactin.F (5'-GTAACAATGCCATGTTCAAT-3') and *bactin.R* (5'-CTCCATCGTGGGCCGCTCTAG-3').

Preparation of Bone Marrow Neutrophils

Mice were sacrificed and the femur and tibia from both hind legs were dissected away from the soft tissue. The tips of the bones were cut off and the bone marrow was flushed with PBS. A single cell suspension was then layered onto 1.08 g/ml Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged ($1000 \times g$, 4°C, 20 minutes). The pelleted cells were recovered and washed in PBS. Red blood cells were eliminated by ammonium chloride/potassium lysis. Cytospin preparations were made with 2×10^5 cells, and stained with the modified Wright Giemsa stain Hema 3 (Fisher Diagnostics). The percentage of neutrophils was determined for each sample and found to be 90 to 95%. Cells were lysed in RIPA with 1% Nonidet P-40 (Calbiochem, La Jolla, CA), 1 mmol/L phenylmethyl sulfonyl fluoride, and Complete protease inhibitor cocktail (Roche). Cellular debris was removed by centrifugation and protein content was assayed using the BCA protein assay reagent kit (Pierce, Rockford, IL).

Western Blot Analysis

Total cell lysate (2.5 μ g) was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and subsequently transferred to an Immobilon P membrane (Millipore, Bedford, MA). *Ncf1* was detected with goat anti-human *Ncf1* (Novus Biologicals, Littleton, CO) followed by horseradish peroxidase-conjugated donkey anti-goat IgG (Jackson Immunoresearch, West Grove, PA). p38 MAP kinase was used as a loading control and detected with rabbit anti-p38 (Cell Signaling Technology, Beverly, MA) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA). Blots were developed using Supersignal West Pico chemiluminescent substrate (Pierce).

Oxidative Burst Assay

Oxidative burst capacity of neutrophils was determined as previously described.¹⁸ Briefly, peritoneal neutrophils were prepared as described (see Recruitment of Peritoneal Neutrophils) and plated in a 96-well flat bottom microtiter plate containing 100 μ g of cytochrome C (Sigma). Cells were maintained at 37°C and incubated in 20 μ l HBSS, 5% fetal calf serum, and 10 mmol/L HEPES alone or 20 μ l HBSS, 5% fetal calf serum, and 10 mmol/L HEPES with either phorbol myristate acetate (PMA) (100 ng/ml) or WKYMVM (5×10^{-6} mol/L) (Bachem, King of Prussia, PA). Triplicate reactions were allowed to proceed for 1 hour while relative production of reactive oxygen species was determined from optical densities at 550 nm using the Spectramax Plus (Molecular Devices, Sunnyvale, CA) at 1-minute intervals.

Sequencing of *Ncf1*

Ncf1 sequence was obtained from genomic DNA in BALB/cAnNCr, C3H/HeNCr, and C57BL/6NCr and from mRNA in C3H and C57BL/6 mice. For mRNA sequence cDNA was made from total RNA purified from spleens using RNeasy kit (Qiagen, Valencia, CA). *Ncf1* was amplified using primers mouse p47.F (5'-CAGCCATGGGGACACCTTCA-3') and mouse p47.R (5'-AGCGCTCACTGCCTCCTCTCAT-3') and sequenced. For genomic DNA sequence the *Ncf1* gene was amplified using three sets of primers derived from GenBank NT_039314. The total length sequenced in each strain was 7242 nucleotides. Product 1 spans University of California Santa Cruz (May 2004 build) coordinates 131953210 to 131954310 and overlapping products 2 and 3 span 131946770 to 131952910. Product one was amplified using the GC-rich PCR System (Roche). Each 50- μ l reaction contained: 100 ng of genomic DNA, 15 pmol/L left (*ncf1.1.left* 5'-CAAGGGAAGGATGTGAGAGG-3') and right (*ncf1.1.right* 5'-CCATGAGGCCGTTGAAGTAT-3') primers, 200 μ mol/L dNTPs, 1 mol/L GC-RICH resolution solution, 1.5 mmol/L $MgCl_2$, 1 $\times$ PCR buffer, and 1 U enzyme mix (*Taq* DNA polymerase in combination with proofreading polymerases). Cycling conditions included an initial denaturation at 95°C for 3 minutes; 10 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes followed by 20 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes + 5 seconds/cycle. Products 2 and 3 were amplified using the Expand Long Template PCR system (Roche). Each 50- μ l reaction contained: 100 ng of genomic DNA, 15 pmol/L left (*ncf1.2.left* 5'-ACTAGGCAATCTGTGCCACG-3'; *ncf1.3.left* 5'-CTTGGATGTCTGCACTGAG-3') and right (*ncf1.2.right* TGCTTTGAGTTGCCTCACTG; *ncf1.3.right* TGGTCTTAGGCAACCTCTGG) primers, 200 μ mol/L dNTPs, 1 $\times$ reaction buffer 2, and 2.6 U *Taq/Pwo* polymerase mix. Cycling conditions included an initial denaturation at 94°C for 2 minutes; 10 cycles at 94°C for 10 seconds, 55°C for 30 seconds, 68°C for 2 minutes followed by 20 cycles at 94°C for 10 seconds, 55°C for 30 seconds, 68°C for 2 minutes + 20 seconds/cycle. Residual primers and dNTPs were removed from the PCR products using a 96-well glass fiber filter (Millipore). The sequence-ready templates were eluted in 120 μ l of sterile water. Amplified products were then sequenced using internal primers (Table 1) in 10- μ l reaction volumes using ABI BigDye Terminator chemistry. Cycling conditions included an initial denaturation at 96°C for 30 seconds; followed by 46 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. On completion of cycle sequencing, 40 μ l of 62.5% ethyl alcohol/1 mol/L potassium acetate (pH 4.5) was added to each reaction, and the sequence plates were centrifuged at 4000 rpm at 4°C for 45 minutes. The samples were resuspended in 15 μ l of sterile H_2O and were electrophoresed on an ABI3700 DNA analyzer prepared with POP-5 capillary gel matrix (ABI, Foster City, CA). Sequence trace files were evaluated using the Phred, Phrap, and Consed programs.²⁶

Table 1. Primers Used for Sequencing *Ncf1*

Primer	Sequence 5' to 3'	Primer	Sequence 5' to 3'
Ncf1.1.J	GAAGACAGAGAGGGAGGCAG	Ncf1.2.R	CGAACTCAGAAATCCGCTT
Ncf1.1.K	AACCAAGGGAAGGATGTGA	Ncf1.2.S	CTGACAAAGGCTGGGAAGAA
Ncf1.1.L	CCGAGCAGACCCAGTGATAG	Ncf1.2.T	GCACACATGCATGAACACAC
Ncf1.1.M	CCAGGAAACCTCCCTGTCTA	Ncf1.3.A	GAGATTCCAGCTGGGCGT
Ncf1.1.N	CCTTGGATGGAAGGCTGA	Ncf1.3.B	ACATCCACACCCACACACAC
Ncf1.1.O	CCTCCTCCCTCCCTCTCTAA	Ncf1.3.C	CAGTCTGGGCAACGTAGTGA
Ncf1.1.P	GCCCTCGGAAATCTCTAGGT	Ncf1.3.D	GAATGGGCTGAAAGCAAGTC
Ncf1.1.Q	GTTCCCTGGTTAAGTGGCAGG	Ncf1.3.E	ACACTTCTAGGTAAGAGATCCATCAGT
Ncf1.1.R	GGATCAGGAGTTCAAAGGCA	Ncf1.3.F	CATGCTCTACCACTGAGCCA
Ncf1.1.T	AGAGAGCAGAGGAGGAGCAA	Ncf1.3.G	GGCTACACTTCCCAGATGACA
Ncf1.2.A	GGCGAGATCCACACAGAGA	Ncf1.3.H	TACCACAAGCACACAGCCTC
Ncf1.2.B	CTCATGGTGACATGGACCT	Ncf1.3.I	CAAGCCTTTGGTCTTCCCTT
Ncf1.2.C	ATACTTCAACGGCCTCATGG	Ncf1.3.J	AACTCTGCGGCTAATGAGGA
Ncf1.2.D	CTGGGAGTGGAGGCAGAGT	Ncf1.3.K	TGCTGTTGAAGAGGACGAGA
Ncf1.2.E	CATGTCACCATGAGGCATTC	Ncf1.3.L	GCAAGGGAAGAAAGCAGATG
Ncf1.2.F	CCAGGTCTTTATGTCTGTTCA	Ncf1.3.M	TCATTAGCCGACAGATTGCTCA
Ncf1.2.G	ACCTGAAACCCTCCCTTCTG	Ncf1.3.N	CTGCAGATACATGGATGGGA
Ncf1.2.H	ACAGTGTAGGTCTGGCCTGG	Ncf1.3.O	GAAACAGCTTGGCCTTTCAG
Ncf1.2.I	CAAGGCAGGAGAATCAGGAG	Ncf1.3.P	CTGTTTCCACACCCACTTT
Ncf1.2.J	ATGATGGCGGATGAGGTTAG	Ncf1.3.Q	TCTGAGGAGTTTCTGGCTG
Ncf1.2.K	TCCTGAGTTCAAATCCACAGC	Ncf1.3.R	GCAGGATGCAAGTGTCAAGT
Ncf1.2.L	TGACCGCTCTCGCTCTTC	Ncf1.3.S	CTTGAAGTTTCCCTTGCCTG
Ncf1.2.M	CCTGCTGTGTCCATTACC	Ncf1.3.T	CAGTTGTAAGGTGTCTCGCAG
Ncf1.2.N	AGAGAAGAAAGTGAGGGTGGC	Ncf1.3.U	CAACAGCGTCCGATTCTC
Ncf1.2.O	TCCATCTCAGACCTATCCCG	Ncf1.3.V	CCAGTCTGTCTTGTGCTGT
Ncf1.2.P	ACATTGCTCACATGGTCCTG	Ncf1.3.W	CTGCGAGCACCTTACAACCTG
Ncf1.2.Q	GTCCCTGCATCTATCTGGA	Ncf1.3.X	ATGGCCAACAGGTTGAGAAA

Statistical Analysis

Statistical analysis was performed using Student's *t*-test or Mann-Whitney *U* (as noted) for two group comparisons and one-way analysis of variance followed by either Tukey's HSD (equal variances assumed) or Tamhane's T2 (equal variances not assumed) post hoc test for three group comparisons. Values of *P* < 0.05 were considered significant.

Results

Bb2Bb3 Regulates Lyme Arthritis Severity

Genetic regulation of Lyme arthritis severity in mice was previously analyzed by QTL assessment of intercross populations between severely arthritic C3H/He and mildly arthritic C57BL/6 or BALB/c mice that had been infected with *B. burgdorferi*. Lyme arthritis was found to be multi-genic with significant linkages identified for six different QTL on five chromosomes.^{11,12} Two separate QTL were initially identified on chromosome 5, one determined by measurement of rear ankle swelling, *Bb2*, and the second by histopathologically assessed arthritis severity, *Bb3*. Composite interval mapping indicated overlap of the arthritis traits and suggested that up to four distinct regulatory loci were present in a region spanning ~36 cM on chromosome 5, from 44 to 80 cM. LOD scores for linkage to this region ranged from 3.5 to 10.2, with the strongest linkage positioned at 72 cM. Congenic lines were developed in which the entire *Bb2Bb3* region of C3H/He was introgressed onto C57BL/6 mice (B6.C3-Bb2Bb3) and

the same region of C57BL/6 was introgressed onto C3H/He mice (C3.B6-Bb2Bb3).

Transfer of the arthritis phenotype was assessed by infection of the congenic mice with 2000 spirochetes of the N40 isolate of *B. burgdorferi*. As shown in Figure 1 and Table 2, both congenic lines developed arthritis of severity that was significantly different from the respective parental C3H/HeN and C57BL/6 strains. *B. burgdorferi* infection in female B6.C3-Bb2Bb3 mice resulted in sig-

Figure 1. Lyme arthritis severity in C3H/HeN, C57BL/6, B6.C3-Bb2Bb3, and C3.B6-Bb2Bb3 mice. Ankle measurements at 4 weeks after *B. burgdorferi* infection were taken as described and used as an assessment of arthritis severity. *Significantly increased (*P* < 0.05; Student's *t*-test) in female B6.C3-Bb2Bb3 (*n* = 13) compared to female C57BL/6 (*n* = 10) mice. †Significantly decreased (*P* < 0.05; Student's *t*-test) in female C3.B6-Bb2Bb3 (*n* = 4) compared to C3H/HeN (*n* = 10) mice. ‡Significantly decreased (*P* < 0.05; Student's *t*-test) in male C3.B6-Bb2Bb3 (*n* = 10) compared to C3H/HeN (*n* = 10) mice.

Table 2. Histological Assessment of Arthritis Severity in *Bb2Bb3* Congenic Mice

	Overall lesion	Neutrophil infiltration	Mononuclear infiltration	Tendon sheath thickness	Reactive/reparative	Total
C57BL/6	1.1 ± 0.77	0.85 ± 1.04	0.15 ± 0.37	0.9 ± 0.85	0.75 ± 1.07	3.45 ± 3.49
B6.C3- <i>Bb2Bb3</i>	2.52 ± 1.08*	1.30 ± 1.19	0.17 ± 0.39	1.96 ± 1.30*	2.35 ± 1.03*	7.87 ± 3.78*
C3.B6- <i>Bb2Bb3</i>	1.86 ± 1.23 [†]	1.0 ± 0.96 [†]	0 ± 0	1.286 ± 0.91 [†]	1.5 ± 1.16 [†]	5.5 ± 3.76 [†]
C3H/HeN	3.3 ± 0.80	1.95 ± 0.76	0.05 ± 0.22	3.05 ± 0.67	2.75 ± 0.97	11.05 ± 2.50

*Significantly increased in B6.C3-*Bb2Bb3* ($n = 24$) when compared to C57BL/6 ($n = 20$) ($P < 0.05$, Mann-Whitney U -test).

[†]Significantly decreased in C3.B6-*Bb2Bb3* ($n = 14$) when compared to C3H/HeN ($n = 20$) ($P < 0.05$, Mann-Whitney U -test).

nificantly greater rear ankle swelling than wild-type C57BL/6 mice (Figure 1). A similar trend of increased ankle swelling was also observed for the male B6.C3-*Bb2Bb3* mice, although statistical significance was not reached when compared with male C57BL/6 mice. This may reflect the greater range of arthritis severity observed in male C57BL/6 mice compared to female C57BL/6 mice. Even more convincing was the finding that both male and female B6.C3-*Bb2Bb3* mice infected with *B. burgdorferi* displayed significantly increased arthritis severity as determined by several parameters of histologically scored arthritis when compared with wild-type C57BL/6 mice (Table 2). Transfer of *Bb2Bb3* from C3H/He mice resulted in increased tendon sheath thickness, reactive/reparative changes, and overall lesion severity as compared with infected C57BL/6 mice. The reciprocal congenic line, C3.B6-*Bb2Bb3*, also revealed transfer of phenotype. Infected C3.B6-*Bb2Bb3* mice developed significantly less ankle swelling and less severe histopathology associated with Lyme arthritis than the C3H/He parent (Figure 1, Table 2). Importantly, the presence of the *Bb2Bb3* locus from C57BL/6 mice protected C3H/He mice from the most severe aspects of arthritis manifestation including neutrophil infiltration, thickness of the tendon sheath, and reactive reparative changes consistent with bone remodeling.

The results presented in Figure 1 and Table 2 provide strong confirmation that *Bb2Bb3* contains regulatory loci for Lyme arthritis. The fact that both congenic lines developed arthritis of intermediate severity compared to that observed for the parental C3H/He and C57BL/6 strains was not surprising as QTL on four other chromosomes also contribute to the difference in Lyme arthritis severity between C3H/He and C57BL/6 mice.¹² Microsatellite-based genotyping confirmed that other *B. burgdorferi* arthritis-associated QTL were not transferred with *Bb2Bb3* during generation of the congenic lines (see Materials and Methods). Thus, the finding of significantly increased arthritis in B6.C3-*Bb2Bb3* and protection in C3.B6-*Bb2Bb3* mice is an extremely important indication of the potency of the QTL on chromosome 5. Furthermore, although *Bb2Bb3* can function partially independent of other QTL, genes in other QTL are necessary for the complete manifestation of arthritis that develops in infected C3H/He mice. The demonstration of a strong effect of *Bb2Bb3* on Lyme arthritis phenotype indicates that one or more genes within this region of chromosome 5 regulate Lyme arthritis severity.

Are There Genetic Polymorphisms in *Ncf1*?

The discovery that *Ncf1* was linked to arthritis severity in four models of rat arthritis and that in mice this gene is located near the highest linkage (LOD score 10.2, 72 cM) in *Bb2Bb3*, prompted us to determine whether alleles of *Ncf1* were responsible for *Bb2Bb3* regulation of Lyme arthritis severity. The C57BL/6 genome sequence is available in public databases; however, this sequence is not available for C3H/He mice. The entire *Ncf1* mRNA sequence including the proximal 5' untranslated region as well as 7000 bp of genomic DNA beginning in the first intron of *Ncf1*, were determined for the substrains of C57BL/6, C3H/He, and BALB/c mice used in our mouse mapping project. As shown in Figure 2, a single polymorphism was identified, in intron 8. This polymorphism consisted of an additional C in a run of nine Cs, positioned 38 bp from a splice site, in the severely arthritic C3H allele as compared with the allele from mice displaying less severe Lyme arthritis, C57BL/6 and BALB/c. Although it is not likely that a SNP in an intron would alter the amino acid sequence of the *Ncf1* protein, there is precedent for alteration in runs of pyrimidines influencing splicing efficiency in mammalian genes, and thereby influencing exon usage.^{27–29} Alterations in exon usage could influence the level of functional *Ncf1* protein produced by phagocytic cells of C57BL/6 or C3H/He mice.

Extensive analysis was performed to detect alterations in splicing or relative expression of 5' and 3' exons of the *Ncf1* gene product. Northern blot analysis failed to detect distinct species of, or quantitative differences in *Ncf1* mRNA from PMNs, macrophages, and splenocytes of

Figure 2. Identification of a single nucleotide polymorphism in intron 8 of the *Ncf1* gene. Genomic and cDNA sequence of *Ncf1* was obtained from C57BL/6, BALB/c, and C3H/HeN mice. A single nucleotide polymorphism was identified 38 bp 5' of exon 9. An asterisk indicates 131,948,193 Mb on chromosome 5 according to the mouse University of California Santa Cruz genome sequence (May 2004 build).

Figure 3. Quantification of Ncf1, in phagocytic cells from C3H/HeN and C57BL/6 mice. **A:** Ncf1 transcripts were quantified in peritoneal neutrophils from C3H/HeN, C57BL/6, and BALB/c mice. Quantitative real-time PCR was performed with primers designed to detect 5' or 3' transcript levels, which were found to be similar in all strains. **B:** Ncf1 protein levels were determined by Western blot analysis in bone marrow neutrophils from several C3H/HeN and C57BL/6 mice.

C3H/He and C57BL/6 mice (data not shown). In addition, RT-PCR with primers designed to span exons surrounding exon 8 failed to reveal novel products that would support allele-specific splicing (data not shown). Quantitative RT-PCR was performed with primers that amplified sequences from the 5' and 3' regions of the cDNA and approximately equivalent levels of product were detected in macrophages and PMNs from C3H/He, C57BL/6, and BALB/c mice (Figure 3 and data not shown). Analysis of the protein level in PMNs and macrophages was performed using commercially available antibody to the carboxyl terminus of Ncf1. Equal protein loading was confirmed by detection of p38 MAP kinase, which is constitutively expressed by phagocytes. Ncf1 protein could be detected by Western blot in lysates from splenocytes, macrophages, and PMNs from C3H/He and C57BL/6 mice and was missing from lysates of *Ncf1*^{-/-} mouse cells, shown for PMNs in Figure 3. However, there was not a difference in the quantity of Ncf1 protein that would explain the greater enzymatic activity we hypothesized the mildly arthritic C57BL/6 mice would have (Figure 3). In fact, cells from C3H/He mice consistently possessed similar or more full-length Ncf1 protein than did cells from C57BL/6 mice (Figure 3 and data not shown). If the intronic SNP identified in the C3H/He allele of *Ncf1* caused an alteration in splicing resulting in a truncated protein we would have expected to see a reduction in

Figure 4. Oxidative burst capacity of PMNs from C3H/HeN, C57BL/6, B6.C3-Bb2Bb3, and C3.B6-Bb2Bb3 mice. PMNs were recruited to the peritoneal cavity of mice using thioglycollate. Cells were stimulated with PMA or the chemotactic peptide WKYMVM and oxidative burst capacity was determined. Total oxidative burst capacity at 20 minutes after stimulation was normalized to the number of PMNs as determined by Hema 3 staining. These results are representative of several similar experiments and error bars indicate $\pm$ 1SE of triplicate measurements. *Significantly different ($P < 0.05$; Student's *t*-test) in C57BL/6 phagocytes when compared to C3H/HeN phagocytes.

full-length protein in cells from C3H/He mice, a reduction in transcript detectable with probes from the 3' region in C3H/He mice, or the appearance of novel exon-specific PCR products from transcripts of C3H/He mice. No such modification was found, indicating the intronic SNP in C3H/He mice was unlikely to influence the structure or amount of Ncf1.

Do Polymorphisms in *Ncf1* Influence NADPH Oxidase Activity?

We next determined if there was a difference in the oxidative burst capacity of PMNs from C3H/He and C57BL/6 mice. PMNs were recruited to the peritoneal cavity for 4 hours with thioglycollate, and incubated with PMA, a strong activating agent for the oxidative burst. Because the percentage of PMNs recovered was highly variable, all values were normalized to the number of PMNs recovered from each animal. In numerous experiments PMNs from C3H/He, BALB/c, and C57BL/6 mice were found to have identical maximal and kinetic oxidative burst responses to PMA (Figure 4 and data not shown). Because PMA is a strong stimulus that activates both protein kinase C and diacylglyceride pathways directly, without involvement of an extracellular signaling receptor, a more physiological stimulus was also tested. A novel chemotactic peptide (WKYMVM) was recently described that induces a more robust oxidative burst than f-MLP in mouse PMNs.³⁰ This synthetic peptide was found to induce a greater oxidative burst in PMNs from C57BL/6 mice than in PMNs from C3H/He mice (Figure 4). This supported the possibility that a reduced oxidative burst could be responsible for the increased arthritis in *B. burgdorferi*-infected C3H/He mice relative to C57BL/6 mice.

To determine whether the polymorphism in *Ncf1* was responsible for the difference in oxidative burst in C3H/He and C57BL/6 mice, congenic mouse lines car-

rying introgressed *Bb2Bb3* were tested. PMNs from B6.C3-*Bb2Bb3* displayed a high oxidative burst, similar to the C57BL/6 parent, whereas PMNs from C3.B6-*Bb2Bb3* responded with a lower oxidative burst, similar to the cells from the C3H/He parent (Figure 4). These results clearly demonstrate that the magnitude of the oxidative burst is determined by the background of the mouse strain and is not influenced by the *Ncf1* allele or any other gene mapping to *Bb2Bb3* of the donor mouse strain. No other subunit of the NADPH oxidase complex maps to the *Bb2Bb3* locus or to any other Lyme arthritis QTL. Therefore, the phenotypic differences in NADPH oxidative burst capacity of PMNs from C3H/He and C57BL/6 mice are not determined by the *Ncf1* allele.

Does the Phagocyte NADPH Oxidase Have a Role in Lyme Arthritis Severity or Host Defense to *B. burgdorferi*?

The finding of van de Loo and colleagues,¹⁹ that NADPH oxidase modulates the severity of zymosan and immune-complex arthritis in mice and that its absence causes a dramatic increase in arthritis severity suggested that mice lacking a subunit of the NADPH oxidase complex might develop more severe arthritis when infected with *B. burgdorferi*. Furthermore, we demonstrated that PMNs from severely arthritic C3H/HeN mice generate a lower oxidative burst than PMNs from the mildly arthritic C57BL/6 mice in response to chemotactic peptide (Figure 4). Although polymorphisms in *Ncf1* do not appear to be responsible for mouse strain differences in the severity of Lyme arthritis in mice, it is likely that other genes do regulate the differences in reactive oxygen intermediate production seen between these two strains. To directly assess the possibility of any involvement of NADPH oxidase in regulating Lyme arthritis severity mice deficient in two different subunits of the NADPH oxidase complex were infected.

Mice with a disruption in either *Ncf1* or *Gp91phox* on the mildly arthritic C57BL/6 background were available, allowing us to determine whether a deficiency in NADPH oxidase activity results in an increase in arthritis severity. *Ncf1* and *Gp91phox* are expressed by phagocytes and lymphocytes and required for the NADPH oxidase activity in these cell types.^{13,14,31} Endothelial cells and vascular smooth muscle cells also express a NADPH oxidase that requires *Ncf1*;^{32–34} however, *Gp91phox* is not required for NADPH oxidase activity in these cells because they express homologs of *Gp91phox*.^{35–37} Testing mice deficient in these two subunits allowed us to determine whether there is a broader requirement for NADPH oxidase in regulating Lyme arthritis.

Ncf1^{−/−} and *Gp91phox*^{−/−} mice did not display an increase in ankle swelling or histopathology associated with arthritis when compared with infected C57BL/6 mice (Table 3). Therefore, generation of reactive oxygen intermediates is not involved in the suppression of Lyme arthritis development in C57BL/6 mice. Caution must be taken in experiments with mice deficient in the NADPH oxidase because they are compromised in the ability to

Table 3. Role of the Phagocyte NADPH Oxidase in Murine Lyme Arthritis

	Δ Ankle measurement (mm)	Overall lesion
Experiment A* [†]		
C57BL/6 (n = 9)	0.22 ± 0.21 [†]	1.89 ± 1.05 [†]
C3H/HeN (n = 10)	0.95 ± 0.16 [†]	3.00 ± 0.82 [†]
<i>Ncf1</i> ^{−/−} (n = 3)	0.23 ± 0.13 [†]	0.77 ± 0.83 [†]
Experiment B* [§]		
C57BL/6 (n = 7)	0.21 ± 0.11	0.57 ± 1.13
<i>Ncf1</i> ^{−/−} (n = 7)	0.39 ± 0.39	1.57 ± 1.51
<i>Gp91phox</i> ^{−/−} (n = 8)	0.33 ± 0.23	1.57 ± 1.51

*The number of mice in each group is indicated in parentheses. Data are from a single experiment and representative of several experiments.

[†]Significantly greater in C3H/HeN when compared to C57BL/6 and C57BL/6 *Ncf1*^{−/−} (*P* < 0.05; analysis of variance) at 4 weeks after infection.

[‡]Infected with 2×10^3 *B. burgdorferi*.

[§]Infected with 1×10^5 *B. burgdorferi*.

control certain pathogens as well as opportunistic microbes. During initial experiments we discovered that several uninfected mice had visible lesions on their ankles and paws and in a single experiment we observed more severe arthritis in infected *Ncf1*^{−/−} mice when compared with wild-type C57BL/6 mice. In subsequent experiments when animals were maintained on antibiotics before infection (see Materials and Methods) the *Ncf1*^{−/−} mice developed arthritis similar to wild-type C57BL/6 mice (Table 3) leading us to conclude that co-infection or other environmental factors beyond our control were responsible for the single anomalous result. Therefore, we conclude that mice deficient in the NADPH oxidase do not display arthritis of increased severity when infected with *B. burgdorferi*. The lack of effect of *Ncf1* and *Gp91phox* disruption on Lyme arthritis was a startling observation because it suggested not only that *Ncf1* could be excluded as a candidate gene in the *Bb2Bb3* locus controlling Lyme arthritis severity, but that the localized generation of reactive oxygen intermediates is not required to suppress Lyme arthritis.

Because generation of the oxidative burst by phagocytic cells is crucial to the host defense against many bacterial pathogens and opportunists in humans and mice^{15–17} we tested the effect of a deficiency in NADPH oxidase on host defense to *B. burgdorferi*. *Ncf1*^{−/−} mice had similar spirochete loads in joints (Figure 5), ears, and hearts (data not shown) as those found in wild-type mice. Although inoculum dose does not affect spirochete loads in wild-type mice,¹⁰ it is possible that the relatively low infectious dose normally used, 2×10^3 spirochetes, was not sufficient to overcome the host defense. When infected with 1×10^5 spirochetes mice lacking either *Ncf1* or *Gp91phox* harbored levels of *B. burgdorferi* similar to those found in wild-type C57BL/6 mice in joints (Figure 5), ears, and hearts (data not shown). The absence of the phagocyte NADPH oxidase did not influence the host defense and we conclude that reactive oxygen intermediates are not involved *in vivo* in the control of *B. burgdorferi*.

Figure 5. Spirochete levels in joint tissues of mice deficient in the phagocyte NADPH oxidase. C57BL/6, C3H/HeN, and *Ncf1*^{-/-} were infected intradermally with 2×10^3 *B. burgdorferi*. To challenge the host defense C57BL/6, *Ncf1*^{-/-}, and *Gp91phox*^{-/-} mice were infected intradermally with 1×10^3 spirochetes. In both cases mice were sacrificed at 4 weeks after infection and *B. burgdorferi* numbers in the joints were determined using real-time quantitative PCR.

Discussion

We have pursued a genetic analysis of the regulation of Lyme arthritis severity in mice with the assumption that pathways involved in the regulation of this inflammatory arthritis will identify common pathways involved in the development of inflammatory pathologies in humans. Several QTL were identified, with highly significant linkage found to the *Bb2Bb3* region of chromosome 5.^{11,12} Development of congenic lines in which the *Bb2Bb3* loci of the parental C3H/He and C57BL/6 mice were introgressed onto the reciprocal background allowed formal assessment of the penetrance of arthritis severity alleles in this model. We found significantly reduced arthritis severity in C3H/He mice carrying the *Bb2Bb3* allele from C57BL/6 mice and a corollary increase in arthritis in C57BL/6 mice carrying the *Bb2Bb3* allele from C3H/He mice. These results, presented in Figure 1 and Table 2, conclusively demonstrate the existence of gene(s) in this region of chromosome 5 that regulate the severity of murine Lyme arthritis. The fact that *Bb2Bb3* could not

completely transfer the mild or severe arthritis is consistent with polygenic control of the Lyme arthritis phenotype.¹¹ However, the relatively robust phenotypic transfer may be indicative of the presence of several genes in the *Bb2Bb3* region that contribute to regulation of arthritis severity, as previously suggested by composite interval mapping.¹² In addition the partial transfer of phenotype is consistent with the presence of four other QTL on different chromosomes that contribute to the most severe arthritis in C3H/He mice and profound difference in severity with C57BL/6 mice.

The recent finding that reactive oxygen intermediates play a suppressive role in both mouse and rat models of rheumatoid arthritis provided a compelling reason to assess the role of reactive oxygen intermediates in the early events of Lyme arthritis.^{18–20} The primary source of reactive oxygen intermediates during infection and inflammation is the phagocyte NADPH oxidase, which is abundantly expressed by neutrophils, to lesser extent in macrophages, and recently appreciated to be expressed by T lymphocytes.^{31,38,39} PMN infiltration is characteristic and essential to the development of both Lyme arthritis and rheumatoid arthritis, suggesting that if the oxidative burst had a protective effect in rheumatoid arthritis it could also play a role in Lyme arthritis.^{21–23} Olofsson and colleagues¹⁸ demonstrated that alleles of *Ncf1*, a subunit of the NADPH oxidase complex, regulate severity in four different rat models of rheumatoid arthritis. In mice the *Ncf1* gene maps to a region within *Bb2Bb3* that is highly associated with regulation of Lyme arthritis severity, with LOD scores up to 10.2.¹² This suggested that alleles of *Ncf1* could be responsible for the difference in Lyme arthritis severity seen between C3H/He and C57BL/6 mice.

Numerous experiments were performed to test the hypothesis that allelic differences in *Ncf1* could regulate Lyme arthritis severity in mice. A single nucleotide polymorphism in the *Ncf1* gene of the C3H/HeN mouse was identified, within intron 8 (Figure 2). However, this intronic polymorphism was not associated with differences in the level of mRNA expression, or with alterations in RNA splicing or exon usage. Furthermore, there was no reduction in the amount of full-length *Ncf1* protein produced by PMNs, macrophages, or splenocytes as determined by Western blot analysis (Figure 3). Although PMNs from C57BL/6 mice had a stronger oxidative burst than PMNs from C3H/He mice in response to the chemotactic peptide WKYMVM, analysis of PMNs from mice congenic for *Bb2Bb3* indicate that this difference was not due to allelic differences in *Ncf1* (Figure 4). Thus allelic differences in *Ncf1* do not regulate NADPH oxidase activity in C3H/He and C57BL/6 mice.

It has also been demonstrated that mice possessing null mutations in either *Ncf1* or *Gp91phox* developed more severe disease in several experimental models of arthritis, including collagen-induced, zymosan-induced, and immune complex-mediated arthritis.^{19,20} We found that deficiency in either the *Ncf1* or *Gp91phox* subunits of the NADPH oxidase complex did not influence the severity of Lyme arthritis (Table 3). These experiments show that a complete deficiency in reactive oxygen intermedi-

ates produced by NADPH oxidase does not affect the severity of Lyme arthritis. Together the results presented here indicate that the transfer of arthritis phenotype by *Bb2Bb3* is not due to *Ncf1*, but rather to allelic differences in other linked gene(s). Although *Ncf1* is not a candidate gene for regulation of Lyme arthritis severity in mice we expect to identify other novel genes in this region that do regulate arthritis development.

The observation that production of reactive oxygen intermediates is not a shared mechanism by which rheumatoid and Lyme arthritis are regulated may be explained by differences in the mechanisms of these diseases. Although rheumatoid and severe Lyme arthritis are characterized by similar cellular infiltrates,^{4,7,21–23} there are important characteristics that suggest they could be regulated by different mechanisms. In rodent models rheumatoid arthritis is absolutely linked to particular alleles of the MHC,^{40,41} requires T cells that recognize self-antigens such as collagen presented by the MHC susceptibility allele,⁴² and involves antibodies to these self-antigens. Lyme arthritis is unique in that it develops in *scid* and *rag* mice,^{43–46} indicating that the adaptive immune response is not required for the initiation of arthritis. In contrast, the role of initiating antibody and/or T cells is well documented in many rodent models of rheumatoid arthritis.^{41,42} Recent studies with collagen-induced arthritis and experimental autoimmune encephalomyelitis have led to the hypothesis that reactive oxygen intermediates suppress T-cell responses in autoimmunity and that this is a major regulatory effect of the phagocyte NADPH oxidase.²⁰ Our finding that reactive oxygen intermediates play no role in regulation of murine Lyme arthritis are consistent with this due to the T-cell independence of this disease.

Autoimmune conditions such as rheumatoid arthritis and multiple sclerosis are characterized by immune responses to self-antigens that lead to clinical disease. Susceptibility is linked to particular alleles of the MHC in humans and is typically required in animal models of these diseases.⁴⁷ However, it is also clear that other genes, in both humans and animal models, modify susceptibility to and severity of disease. Although the presence of self-reactivity during clinically relevant disease is well established, the events leading to the development of autoimmunity have not been defined. Lyme arthritis is an acute inflammatory reaction to invasion of joint tissue by the spirochete *B. burgdorferi*; however, chronic, treatment-resistant Lyme arthritis described by Steere and colleagues^{6,8,9} is thought to be driven by acquired host defenses and linked to the same MHC susceptibility alleles as rheumatoid arthritis. Therefore, analysis of the mechanisms controlling the development of acute Lyme arthritis may provide clues to the transition from an inflammatory process driven by an infectious agent into the development of autoimmunity. Autoimmune conditions are complex, multistage diseases influenced by both environmental and genetic factors. Thus, it may require the use of several different animal models that represent different temporal stages and environmental triggers to deter-

mine the genetic elements responsible for controlling the susceptibility and severity of disease. Studying the genetic regulation of Lyme arthritis in mice may not only provide insight into regulation of acute inflammatory responses, but also into the conditions that set the stage for the development of autoimmunity.

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