Macrophage p38 Mitogen-Activated Protein Kinase Activity Regulates Invariant Natural Killer T-Cell Responses During *Borrelia burgdorferi* Infection

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The interaction of macrophages with infectious agents leads to the activation of several signaling cascades, including mitogen-activated protein (MAP) kinases, such as p38. We now demonstrate that p38 MAP kinase-mediated responses are critical components to the immune response to *Borrelia burgdorferi*. The pharmacological and genetic inhibition of p38 MAP kinase activity during infection with the spirochete results in increased carditis. In transgenic mice that express a dominant negative form of p38 MAP kinase specifically in macrophages, production of the invariant natural killer T (iNKT) cell-attracting chemokine MCP-1 and of the antigen-presenting molecule CD1d are significantly reduced. The expression of the transgene therefore results in the deficient infiltration of iNKT cells, their decreased activation, and a diminished production of interferon γ (IFN-γ), leading to increased bacterial burdens and inflammation. These results show that p38 MAP kinase provides critical checkpoints for the protective immune response to the spirochete during infection of the heart.

Macrophages play a critical role in the defense against infectious microorganisms. These phagocytic cells detect pathogens via pattern recognition receptors, such as Toll-like receptors (TLRs), scavenger receptors, and integrins [1–3]. This interaction results in the initiation of an array of signaling pathways, leading to the activation of the transcription factor NF-κB, as well as mitogen-activated protein (MAP) kinases, such as p38 [4, 5]. The end result is the production of proinflammatory factors, internalization and degradation of the infecting agent, and the increased ability to present antigen to CD4+ T and invariant natural killer T (iNKT) cells [6–8].

Signal transduction via MAP kinases is important in a variety of cellular responses. Several MAP kinase signal transduction pathways have been defined in mammalian cells [9, 10] that are activated by phosphorylation on Thr and Tyr residues by dual-specificity MAP kinases [11]. In mammalian cells, p38 MAP kinase is activated by MKK3 and MKK6 [12–15] and can be triggered by multiple stimuli, such as proinflammatory cytokines (eg, interleukin 1β and tumor necrosis factor), Toll-like receptor (TLR) stimulation, and physical-chemical changes in the extracellular milieu caused by environmental stress [11, 16–18]. The kinase is a selective target for pyridinyl imidazole [19] and other synthetic molecules [20]. Several clinical trials have tested the use of these drugs for the treatment of, among others, inflammatory neural disorders and rheumatoid arthritis [21–23]. The potential clinical use of p38 MAP kinase inhibitors is based on their ability to inhibit proinflammatory responses. However, the kinase...
also regulates other cellular processes [24], including those important for controlling infection.

*B. burgdorferi* is the causative agent of Lyme disease, a prevalent vector-transmitted infection that is endemic in areas of the United States. Among the symptoms occurring as a result of infection with *B. burgdorferi*, Lyme arthritis and carditis may present to varying degrees in patients infected with the spirochete. The cellular infiltrate in the inflamed joints and hearts of patients and mice experimentally infected with the spirochete are distinct: neutrophils are predominant in arthritis, while carditis is characterized by a macrophage infiltrate at the base of the heart, surrounding the aortic valve [25, 26]. Spirochete colonization of the heart results in early infiltration of macrophages and proinflammatory cytokine production in response to spirochetal antigens [25, 27, 28]. p38 MAP kinase activity controls the proinflammatory response to *B. burgdorferi* in phagocytic cells by regulating the activation of NF-κB [17]. p38 MAP kinase also regulates other cellular processes [24], including those important for controlling infection.

**METHODS**

**Generation of cd11b-dnp38 Transgenic Mice**

The *cd11b-dnp38* MAP kinase transgene was constructed using the *cd11b* promoter extending from base pairs +1704 to +83 and included 83 base pairs of the 5’ untranslated region, extending up to the ATG codon (kindly provided by Dr. Daniel G. Tenen [30]). The plasmid also contains a 2.1-kb fragment of the polyadenylation signals and intron sequences of the human growth hormone [31] (Figure 2A). The *dnp38* gene was inserted between both sequences by digestion with *Hind*III and *XbaI*. The transgene was digested with *Hind*III and *NolI* and purified by electroelution prior to microinjection into fertilized eggs. The microinjected fertilized eggs were then implanted into pseudopregnant females. Founders were identified by slot blot, using a *BamHI-SacI* 0.5-kb fragment from the human growth hormone gene (*hGH*), and by polymerase chain reaction (PCR), using the primers (5’-AGG ATC CCA AGG CCC AAC TCC-3’ and 5’-CTC CTT AGT CTC CTC CTC TTA T-3’). The transgenic (Tg) mice have been backcrossed into the C3H/HeN background for at least 10 generations to establish stable Tg mouse lines. The experiments described herein have been obtained using 2 lines of Tg mice (171 and 178). The expression of the transgene in different tissues and bone marrow–derived macrophages (BMMs) was determined by reverse-transcription PCR (RT-PCR), using the primers 5’-GCC TTT GCA CAT GCC TAC TTT GC-3’ and 5’-GAG GGA GGT CTG GGG GGT TTT CG-3’.

**Infection With B. burgdorferi and Evaluation of Joint and Cardiac Inflammation**

Groups of 5–6 mice were infected by subcutaneous injection with 10⁶ *B. burgdorferi* in the midline of the back, as previously described [29]. At sacrifice, the mice were analyzed for inflammation by histological evaluations of arthritis and carditis. Joints and hearts (cut in half across the atria and ventricles) were fixed in 10% formalin and stained with hematoxylin and eosin. The joints were also decalcified. Signs of arthritis were evaluated as described previously [32] on the basis of assessment of histological parameters, such as exudation of fibrin and inflammatory cells into the joints, alteration in the thickness of tendons or ligament sheaths, and hypertrophy of the synovium [33–35]. Signs of carditis were evaluated on the basis of the cardiac inflammatory infiltrate [33, 36]. Inflammation was blindly scored on a scale of 0 (no inflammation), 1 (mild inflammation), 2 (moderate inflammation), or 3 (severe inflammation). The Institutional Animal Care and Use Committee at the University of Massachusetts–Amherst approved all procedures involving animals.

**Determination of Bacterial Burdens**

The number of spirochetes in skin (ear) and heart tissue was determined by real-time PCR, using primers specific for the *recA* gene (5’-GTG GAT CTA TTG TAT TAG ATG AGG CTC TCG-3’ and 5’-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3’) [37] and the fluorescent DNA dye SYBR Green (Roche, Nutley, NJ). Total DNA was extracted using the Qiagen tissue kit in accordance with the manufacturer’s instructions (Qiagen, Valencia, CA). PCR conditions were as follows: forty 30-second cycles of denaturing (at 95°C) and annealing/extension (at 60°C). Use of these conditions permits detection of approximately 10 spirochetes per picogram of total DNA [38]. The number of spirochetes in each sample were standardized to micrograms of total DNA with the use of primers corresponding to glyceraldehyde 3-phosphate dehydrogenase (5’-CCA TCA CCA TCT TCC AGG AGG GAG-3’ and 5’-CAC AGT CTT CTT GGT GGC AGT GAT-3’) by real-time PCR [38].
Inhibition of p38 MAP Kinase During Infection With B. burgdorferi

The pharmacological inhibitor SB203580 (InvivoGen, San Diego, CA) was resuspended in dimethyl sulfoxide (DMSO), followed by dilution in sterile phosphate-buffered saline (PBS). C3H/HeN mice were administered SB203580 (1 mg/kg) or vehicle (PBS, DMSO) by intraperitoneal injection, in a 200 μL volume, every other day beginning the day of infection with B. burgdorferi [29]. Mice were sacrificed at 2 weeks of infection.

Determination of Expression Levels of cd1d, inkt, mcp-1, and ifny in Cardiac Tissue

RNA was extracted from half of the heart by the isothiocyanate method, following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The RNA was treated with DNase I (Promega, Madison, WI), and reverse transcribed using oligo RT primers (Promega) and Moloney-murine leukemia virus reverse transcriptase (Promega) to generate complementary DNA. Real-time PCR was then performed using the primers for cd1d (5′-GAC ACC TGC CCC CTA TTT GT-3′ and 5′-TGG CTT CTC TCT TAG GTC-3′), inkt (5′-CAC CCT GCT GCA GAT TTA-3′ and 5′-GCC TCA CAA AAC CAA ATG AGA TCA GAA CC-3′), ifny (5′-GCG TTA ATT CAC ACC-3′ and 5′-GGAG GCT GTG GGT TGT GCC CC-3′), mcp-1 (5′-CGG AAC CAA ATG AGA TCA GAA CC-3′ and 5′-GCT GCA GAT TTA CGG GTC AAC TTT-3′), and actin (5′-GAT GAT GCC CCG GCT GTA TTC-3′ and 5′-TCT GCT GTC ACC-3′) in an Mx3005P QPCR System (Stratagene, La Jolla, CA). Fold-induction of the genes were calculated relative to actin values, using the 2-ΔΔCt method.

Generation of BMMs

Murine BMMs were generated as described elsewhere [33]. Cells were collected from the femoral shafts by flushing with 1 mL of cold Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS) and antibiotics. The cell suspensions were dispersed of cell clumps, treated with ACK lysis buffer, and cultured with 20% L929 cell supernatant. The cell suspensions were washed and resuspended in 1 mL of cold Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. The cell suspensions were dispersed of cell clumps, and 1 mL of cold RPMI medium supplemented with 5% DMSO was added. The cells were washed and resuspended in 100 mm × 15 mm Petri dishes (Fisher Scientific, Pittsburgh, PA) for 8 days. Following incubation, nonadherent cells were eliminated, and adherent macrophages were scraped, counted, and prepared for analysis.

Phagocytosis Assay

GFP-expressing B. burgdorferi, strain 297 (Bb914 [39]), were incubated with BMMs in antibiotic-free medium at a multiplicity of infection (MOI) of 10. To distinguish between binding and internalization of spirochetes, the cells were incubated at 4°C and 37°C in parallel. The incubation at 4°C results in the binding but not internalization of spirochetes [40]. After the incubation, the cells were extensively washed to eliminate surface-bound spirochetes and were analyzed by flow cytometry.

Cytokine and B. burgdorferi–specific Immunoglobulin G (IgG) Enzyme-Linked Immunosorbant Assay (ELISA)

The levels of IFN-γ produced ex vivo by splenocytes following injection of cd11b-dnp38 mice and negative littermate controls (NLCs) with α-galactosylceramide (αGalCer) were determined by capture ELISA, according to manufacturer’s recommendations, using the BD optEIA IFN-γ kit II (BD Biosciences, San Jose, CA). MCP-1 and interleukin 6 (IL-6) levels produced by BMMs were detected using the BD optEIA MCP-1 and IL-6 kits, respectively (BD Biosciences).

The analysis of B. burgdorferi–specific sera IgG levels was determined by coating 96-well plates with 0.5 μg/mL of a B. burgdorferi lystate incubated with serial 2-fold dilutions starting at 1/100, followed by incubation with HRP-conjugated anti-mouse IgG (BD Biosciences) (1/10 000 dilution). The reactions were developed using 1-component tetramethylbenzidine substrate and were stopped with tetramethylbenzidine stop solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Flow Cytometry

A total of 10⁶ splenocytes were incubated for 5 minutes with Fc block (BD Biosciences) and for 45 minutes at 4°C with F4/80 APC (BM8, eBioscience, San Diego, CA). The cells were washed and resuspended in PBS plus 1% FCS for analysis by flow cytometry, using an LSR II flow cytometer (BD Biosciences). The data were analyzed with FlowJo for Mac, version 8.6 (Tree Star, Ashland, OR).

To evaluate the surface expression in BMMs, 10⁶ cells were incubated for 5 minutes with Fc block and for 45 minutes at 4°C with TLR2 PE (6C2, eBioscience), CD11b PE (M1/70, BD Biosciences), or CD1d PE (1B1, BD Biosciences). The macrophages were washed and analyzed by flow cytometry.

Cytokine Induction by αGalCer

A suspension of αGalCer (Enzo Life Sciences, Farmingdale, NY) was prepared in DMSO, followed by dilution in PBS plus 0.5% Tween-20. αGalCer (100 ng/g) or vehicle (PBS plus 0.5% Tween-20) was administered by intraperitoneal injection in a 100 μL volume. NLC and cd11b-dnp38 mouse splenocytes were harvested after 12 hours and cultured in Click’s media (Sigma Chemical, St. Louis, MO) supplemented with 10% FCS (HyClone Thermo Scientific, Waltham, MA), 1% penicillin/streptomycin (Lonza, Allendale, NJ), L-glutamine (Sigma), and 4.4 μL/L of 2-metcaptoethanol (Sigma) for 72 hours. Supernatants were collected and analyzed by ELISA for cytokine production.

Statistical Analysis

The results are presented as means ± standard error. Significant differences between means were calculated with the
Student t test. P values of ≤ 0.05 were considered statistically significant.

RESULTS

Inhibition of p38 MAP Kinase During Infection With B. burgdorferi Results in Increased Carditis

p38 MAP kinase is an important regulator of innate and adaptive immune responses [24]. To assess the specific role of p38 MAP kinase activity in the development of acute inflammation during infection with B. burgdorferi, we first investigated pathology in mice that had been treated with the specific pharmacological inhibitor of p38 MAP kinase, SB203580, during infection. Although the arthritis severity in the SB203580-treated mice was reduced compared with that in control-treated mice, the difference was not significant (P > 0.05, by the Student t test; Figure 1A). However, SB203580 treatment resulted in a significant increase in the severity of cardiac inflammation, compared with vehicle control–treated, B. burgdorferi–infected mice (Figure 1A and 1B).

Development of Macrophages and Their Homeostasis Is Not Affected in cd11b-dnp38 Transgenic Mice

Macrophages are the main infiltrating cell in the inflamed heart during infection with B. burgdorferi [27]. Because p38 MAP kinase inhibition reduced B. burgdorferi–induced carditis, we speculated that p38 MAP kinase activity in macrophages negatively regulates cardiac inflammation. We thus generated Tg mice expressing a mutant of p38 MAP kinase that has been previously shown to act as a dominant negative [31], expressed under the control of the CD11b promoter to target its expression to macrophages. The mice were backcrossed to the C3H/HeN background for >10 generations. The transgene was detected in the lung, thymus, and spleen (Figure 2A). BMMs were also tested by RT-PCR for the expression of the dnp38 transgene. The transgene was readily detected in BMMs generated from the Tg mice (Figure 2A).

The cd11b-dnp38 Tg mice developed normally and were born at the expected Mendelian ratio. They showed no signs of gross abnormalities. The expression of the transgene did not affect macrophage generation or homeostasis, as evidenced by flow cytometric analysis of splenic F4/80+ cells (Figure 2B). Macrophage percentages were similar in the Tg and NLC mice (Figure 2B). The levels of expression of CD11b and TLR2 were also equivalent in dnp38-Tg and NLC BMMs (Figure 2C). However, the stimulation of Tg BMMs with B. burgdorferi resulted in a significant reduction in the production of IL-6, compared to NLC BMMs (Figure 2D). Overall, these results show that the expression of the transgene does not affect macrophages development and homeostasis but regulates the induction of cytokines in response to B. burgdorferi stimulation.

Increased Heart Pathology in cd11b-dnp38 Transgenic Mice Infected With B. burgdorferi

cd11b-dnp38 Tg mice were infected with B. burgdorferi and analyzed for cardiac and joint inflammation after 2 weeks of infection. Similar to the results obtained with SB203580, no significant differences were observed in arthritis severity between Tg and NLC mice (Figure 3A). In contrast, the degree of carditis was significantly higher in the Tg animals as compared to the controls (Figure 3A).

We next determined spirochete levels in skin and heart tissue to assess the ability of the Tg mice to control infection. While the number of spirochetes in the ear tissue was equivalent between Tg and NLC mice (Figure 3B), the number of
B. burgdorferi in the infected hearts of the Tg animals were significantly higher (Figure 3C). To determine whether the higher spirochetal numbers in the Tg mice were due to a differential antibody response, we measured the antibody titers specific for B. burgdorferi in the infected Tg mouse sera. B. burgdorferi–specific sera IgG levels were indistinguishable between Tg and NLC infected mice (Figure 3D). We also determined the capacity of Tg BMMs to phagocytose B. burgdorferi to assess whether p38 MAP kinase regulates the capacity to eliminate the spirochete. BMMs generated from Tg mice internalized GFP-expressing B. burgdorferi as efficiently as NLC BMMs (Figure 3E), suggesting that the expression of the dnp38 transgene did not affect their intrinsic phagocytic capacity.

Infection of cd11b-dnp38 Tg Mice With B. burgdorferi Results in Reduced Levels of ifnγ Gene Expression in the Heart

The production of IFN-γ by iNKT cells in the heart of infected mice increases the phagocytic activity of CD11+ macrophages, resulting in enhanced bacterial clearance and reduced cardiac inflammation [8]. We measured iNKT cell infiltration of infected hearts by quantitative real-time PCR. Infected cd11b-dnp38 Tg mice had significantly fewer iNKT cells in the heart as compared to infected NLCs (Figure 4A). Uninfected mice did not contain iNKT cells in the heart, as reported elsewhere [8]. In correlation, the levels of ifnγ messenger RNA (mRNA) detected in the hearts of the infected mice were significantly reduced in the Tg mice (Figure 4B), confirming that iNKT cells are a significant source of the cytokine [8].

The migration of iNKT cells to sites of infection or tumor implantation is governed by the local production of chemokines. The chemokine MCP-1 (ie, CCL-2) has been implicated in the translocation of iNKT cells to the lungs upon infection with Cryptococcus neoformans, as well as to tumors of diverse etiology [41–43]. We therefore analyzed the level of expression of the mcp-1 gene in the hearts of Tg and NLC infected mice. The levels of mcp-1 mRNA were significantly lower in B. burgdorferi–infected, Tg mice as compared to NLC mice (Figure 4C). Furthermore, BMMs in Tg mice secreted significantly lower basal levels of MCP-1 as compared to BMMs in NLC mice, as well as in response to B. burgdorferi stimulation in vitro (Figure 4D). These results show that p38 MAP kinase regulates the expression of MCP-1, the infiltration of iNKT cells to the heart, and the production of the protective cytokine IFN-γ.
Expression of the dnp38 Transgene Results in Reduced Levels of CD1d in Macrophages

IFN-γ increases the expression of the antigen-presenting molecule CD1d in macrophages [8], leading to a positive feedback loop that further increases antigen presentation by CD1d+ macrophages. We analyzed the expression of CD1d in the cardiac tissue upon infection of the Tg mice. The expression levels of cd1d were significantly lower in the Tg mice, compared with the NLC mice (Figure 5A). To determine whether the lower expression levels of cd1d were due to the lower levels of IFN-γ in the infected tissue or, alternatively, due to p38 MAP kinase regulation of antigen-presenting molecule expression, we determined surface CD1d levels in Tg and NLC BMMs. The analysis of CD1d levels in BMMs by flow cytometry showed reduced surface levels in dnp38 macrophages (Figure 5B), indicating that p38 MAP kinase regulates cd1d expression, as reported elsewhere [44].

We next assessed the functional effect of the expression of the dnp38 transgene in macrophages on the ability of iNKT cells to respond to the prototypical antigen, αGalCer. Groups of Tg and NLC mice were injected with αGalCer, and 12 hours later the level of IFN-γ produced by whole splenocytes was assessed ex vivo without further stimulation. The levels of INF-γ induced by αGalCer in Tg and NLC infected mice were significantly reduced as compared to control cells (Figure 5C). Overall, these results demonstrated that p38 MAP kinase regulates the expression of CD1d and the ability of macrophages to present antigen and induce the activation of iNKT cells.

DISCUSSION

Cardiac inflammation during infection with B. burgdorferi involves the migration and activation of macrophages and other...
p38 MAP kinase regulates the expression of IFN-γ by iNKT cells in response to αGalCer through a mechanism that involves the kinase MNK1 and the elongation factor eIF-4e [47]. We now demonstrate another level of control of iNKT cell activation controlled by the kinase through the regulation of the production of the chemokine MCP-1, as well as through antigen presentation mediated by CD1d. The results using the specific p38 MAP kinase inhibitor SB203580 are consistent with a central role of the kinase in the control of cardiac inflammation during infection with the spirochete. Importantly, however, these data indicate that the activity of the MAP kinase in infiltrating macrophages critically regulates both the infiltration and activation of NKT cells.

The recruitment of iNKT cells to sites of inflammation or tumors occurs through the expression of chemokines. Several studies have shown that the chemokine MCP-1 (ie, CCL-2) regulates the infiltration of these cells to inflamed tissues and tumors [41–43], although MCP-1 has been classically associated with the migration of monocytes [48]. Our results show that in response to the spirochete, macrophages produce MCP-1 in a p38 MAP kinase–dependent manner. Thus, dnp38 BMMs produce significantly lower levels of the chemokine in vitro. Importantly, the expression of mcp-1 in the infected hearts of Tg mice is significantly reduced as compared to that in controls. Overall, our results support a model in which the colonization of B. burgdorferi–infected cardiac tissue by macrophages results in p38 MAP kinase–dependent production of MCP-1 and iNKT recruitment. Our results also show that p38 MAP kinase also regulates CD1d expression and antigen presentation to iNKT cells, resulting in the production of the protective cytokine IFN-γ. p38 MAP kinase therefore plays a critical role during the development of the immune response to B. burgdorferi.

**Notes**

**Acknowledgments.** We are grateful to Daniel G. Tenen for providing the cd11b promoter.

**Financial support.** This work was supported by a National Institutes of Health grant (AR 048265 to J. A.), a UMass Amherst Faculty Research Grant (to J. A.), and a fellowship from the American Heart Association to (C. M. J. O.).

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
References


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