

Role of Adrenomedullin in Lyme Disease[▽]

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***Borrelia burgdorferi* stimulates a strong inflammatory response during infection of a mammalian host. To understand the mechanisms of immune regulation employed by the host to control this inflammatory response, we focused our studies on adrenomedullin, a peptide produced in response to bacterial stimuli that exhibits antimicrobial activity and regulates inflammatory responses by modulating the expression of inflammatory cytokines. Specifically, we investigated the effect of *B. burgdorferi* on the expression of adrenomedullin as well as the ability of adrenomedullin to dampen host inflammatory responses to the spirochete. The concentration of adrenomedullin in the synovial fluid of untreated Lyme arthritis patients was elevated compared with that in control osteoarthritis patient samples. In addition, coculture with *B. burgdorferi* significantly increased the expression of adrenomedullin in RAW264.7 macrophages through MyD88-, phosphatidylinositol 3-kinase (PI3-K)-, and p38-dependent signaling cascades. Furthermore, the addition of exogenous adrenomedullin to *B. burgdorferi*-stimulated RAW264.7 macrophages resulted in a significant decrease in the induction of proinflammatory cytokines. Taken together, these results suggest that *B. burgdorferi* increases the production of adrenomedullin, which in turn negatively regulates the *B. burgdorferi*-stimulated inflammatory response.**

Borrelia burgdorferi is the causative agent of Lyme disease, the most common vector-borne disease in the United States. During infection of a mammalian host, *B. burgdorferi* disseminates from the site of entry to colonize distal tissues throughout the body, including the nervous system, heart, and joints. In each of these sites, the interaction of *B. burgdorferi* with the host results in the induction of an inflammatory response. This inflammatory response is important for the control and clearance of the infection, but if left unchecked, inflammation damages the host tissue and causes the clinical manifestations of Lyme disease, including neuroborreliosis, carditis, or arthritis. To avoid an overexuberant and potentially damaging inflammatory response, host cells employ multiple mechanisms for controlling inflammation.

Adrenomedullin is a host factor that negatively regulates inflammation. It is expressed by numerous cells as a proadrenomedullin peptide, which is further processed to yield two mature and active peptides, proadrenomedullin N-terminal 20 peptide (PAMP) and the 52-amino-acid adrenomedullin (34). Adrenomedullin is constitutively secreted from cells in its immature form (26, 51) and is activated after secretion by amidation of its C terminus (33).

Adrenomedullin expression is induced by several bacterial pathogens (3, 30, 57) and by inflammatory environments (22, 23, 36, 53). Adrenomedullin possesses immunomodulatory ac-

tivities which have been demonstrated to downregulate inflammatory processes in a variety of different models, including models of arthritis. For example, the administration of exogenous adrenomedullin protects mice from arthritis in the collagen-induced murine model of rheumatoid arthritis (18). In addition, adrenomedullin has been detected in the synovial fluid of rheumatoid arthritis patients at concentrations significantly higher than that in the less inflammatory synovial fluid of osteoarthritis patients (14, 39, 43). A significant portion of the adrenomedullin peptides detected in these synovial fluid samples are active, as demonstrated by the inhibition of interleukin-6 (IL-6) secretion from the patients' synoviocytes *in vitro* (43).

Because adrenomedullin production is increased by bacterial pathogens and regulates inflammatory responses, we hypothesized that adrenomedullin may be a mechanism by which the mammalian host controls the inflammatory response induced by *B. burgdorferi*. In this report, we show that adrenomedullin expression is increased by macrophages cocultured with *B. burgdorferi* as well as in the synovial fluid of Lyme disease patients. We propose that adrenomedullin may regulate the host response by significantly decreasing the expression of *B. burgdorferi*-induced inflammatory mediators.

MATERIALS AND METHODS

Cell cultures and reagents. RAW264.7 macrophages were grown and maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc.) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin-streptomycin (Mediatech, Inc.).

Bone marrow-derived macrophages from C57BL/6 mice and MyD88^{-/-} mice backcrossed to C57BL/6 for 12 generations were recovered from mouse femurs

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and differentiated as described previously (48, 49). Briefly, bone marrow cells were flushed from femurs with DMEM and cultured on plastic petri dishes for 5 to 7 days in DMEM containing 20% FBS, 30% L929 cell conditioned medium, and 1% penicillin-streptomycin. Differentiated macrophages were then seeded in 24-well plates for experiments. The procedures used for these studies were reviewed and approved by the Tufts University Institutional Animal Care and Use Committee.

Clonal isolates of infectious, low-passage *B. burgdorferi* sensu stricto (strain N40, clone D10E9) were cultured in Barbour-Stoenner-Kelley (BSK II) medium at 37°C as previously described (6).

Recombinant, mature, amidated rat adrenomedullin (American Peptide) was resuspended in endotoxin-free water and stored in small aliquots at -80°C. In cell culture experiments, adrenomedullin was added at a concentration of 100 nM, which was chosen based on prior studies (30, 36, 41, 56). This concentration of adrenomedullin does not affect the viability of RAW264.7 macrophages, as determined by trypan blue exclusion assay.

Patients. Synovial fluid from patients with untreated Lyme arthritis, osteoarthritis, or rheumatoid arthritis was used for these studies. The conduct of this study was reviewed and approved by the Tufts Medical Center Investigational Review Board. Synovial fluid was obtained from 14 patients with untreated Lyme arthritis. These samples have been previously described (37, 38). All patients met the Centers for Disease Control and Prevention clinical criteria for the diagnosis of Lyme disease. They were infected in the northeastern United States and had monoarticular or oligoarticular arthritis affecting at least one knee accompanied by a positive immunoglobulin G Western blot test for Lyme disease interpreted according to the Centers for Disease Control and Prevention/Association of State and Territorial Public Health Laboratory Directors (CDC/ASTPHLD) criteria (12). All 14 patients had a positive PCR test for *B. burgdorferi* DNA in synovial fluid, performed as described by Nocton et al. (44). The mean duration of symptoms at the time of sample collection was 2.2 years (range, 6 months to 6 years).

Synovial fluid from rheumatoid arthritis and osteoarthritis patients was collected from knees of patients diagnosed with rheumatoid arthritis or osteoarthritis by a board-certified rheumatologist and met clinical and/or laboratory criteria for their diagnosis. All specimens were stored at -80°C until use.

Radioimmunoassay. The concentration of adrenomedullin in synovial fluid was determined by radioimmunoassay (Bachem) following the manufacturer's instructions with the following modification: due to the small volume of Lyme arthritis synovial fluid available for use in this assay, the volumes of all samples and reagents were reduced by 5-fold. Briefly, undiluted synovial fluid was incubated with antiadrenomedullin antibodies in 12- by 75-mm polypropylene tubes (Evergreen Scientific) overnight at 4°C. Iodinated adrenomedullin tracer was added to each tube, and the mixture was incubated overnight at 4°C. The adrenomedullin-antibody complexes were immunoprecipitated using secondary antibodies and normal rabbit serum. These pellets were read with a Micromedic gamma counter (Micromedic Systems).

Chemical inhibitors. Chemical inhibitors were added to RAW264.7 macrophages 2 h prior to coculture with *B. burgdorferi*. Ly294002 was purchased from EMD Biosciences/Calbiochem and resuspended in dimethyl sulfoxide (DMSO) following the manufacturer's instructions. SB203580 was purchased from EMD Biosciences/Calbiochem and resuspended in DMSO following the manufacturer's instructions. Cycloheximide was purchased from Sigma-Aldrich and resuspended in endotoxin-free water following the manufacturer's instructions. The concentrations of Ly294002 (10 μ M) and SB203580 (3 μ M) were previously published (8, 49), and these inhibitors had no visible cytotoxic effects on the RAW264.7 cell cultures, as described previously (8, 49). Cycloheximide was added to cell cultures at 5 μ M and had no effect on cell viability as determined by trypan blue exclusion assay.

qRT-PCR. RNA was extracted from RAW264.7 macrophages with TRIzol (Invitrogen) following the manufacturer's instructions. RNA was resuspended in water containing RNaseOut recombinant RNase inhibitor (Invitrogen). RNA was treated with DNase I with the Turbo DNA-free kit (Ambion). cDNA was synthesized with the ImPromII kit (Promega) following the manufacturer's instructions. Quantification of cDNA was performed by quantitative real-time PCR (qRT-PCR) (iCycler, Bio-Rad) with the iQ SYBR green Supermix (Bio-Rad). The cycling parameters were 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primers used to detect tumor necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1), and CXCL-2 cDNA were published previously (7). The sequences of the forward (-F) and reverse (-R) primers used to detect interleukin-6 (IL-6), IL-1 β , β -actin, and adrenomedullin are as follows: IL-6-F, 5'-GACTTCACAGAGGATACCA C-3'; IL-6-R, 5'-TATCCAGTTTGGTAGCATCC-3'; IL-1 β -F, 5'-TGGACCTC TGCCCTCTGGAT-3'; IL-1 β -R, 5'-CATCGTGCACATAAGCCTCGTT-3';

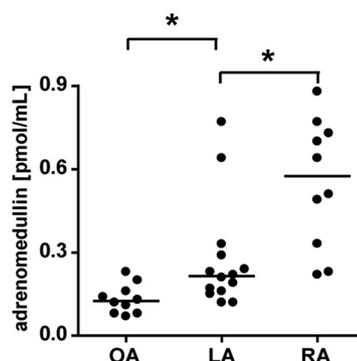


FIG. 1. Adrenomedullin concentrations are elevated in the synovial fluid of patients with untreated Lyme arthritis. Synovial fluid was collected from patients with osteoarthritis (OA), untreated Lyme arthritis (LA), or rheumatoid arthritis (RA). The concentration of adrenomedullin in these samples was measured by radioimmunoassay. Horizontal bars represent the median of each group. *, $P = 0.005$.

β -actin-F, 5'-GTGCGTGACATCAAAGAGAAGC-3'; β -actin-R, 5'-GATGCC ACAGGATTCCATACCC-3'; adrenomedullin-F, 5'-CACCTGATGTTATTG GGTTC-3'; and adrenomedullin-R 5'-TTAGCGCCCACTTATCCACT-3'. Expression of target genes was referenced to expression of β -actin. Calculations of expression were normalized by the cycle threshold ($\Delta\Delta C_T$) method, in which the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$, where C_T is the cycle number of the detection threshold.

ELISA. Supernatants were collected 6 h poststimulation. TNF- α , IL-6, and IL-1 β were measured with the DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) following the manufacturer's instructions.

Statistical analysis. For radioimmunoassay measurements, the median adrenomedullin concentration is represented by a horizontal line. For qRT-PCR and ELISAs, relative cytokine expression/secretion is reported, with error bars representing the standard error of the mean of at least three independent experiments. Statistical significance for all assays was determined using Mann-Whitney U analysis.

RESULTS

Adrenomedullin concentrations are elevated in the synovial fluid of patients with untreated Lyme arthritis. Previous reports have demonstrated that adrenomedullin is produced in response to inflammatory stimuli (22, 23, 35, 53). Because Lyme arthritis is an inflammatory arthritis, we sought to determine the concentration of adrenomedullin in the synovial fluid of Lyme arthritis patients. Synovial fluid was collected from the joints of patients presenting with either untreated Lyme arthritis or the noninflammatory osteoarthritis. The use of a noninflammatory form of arthritis as the negative control was necessary because the volume of synovial fluid in healthy joints is too small to be collected in sufficient quantities. The concentration of adrenomedullin in these synovial fluid samples was measured by radioimmunoassay. The median concentration of adrenomedullin in Lyme arthritis synovial fluids (0.22 pmol/ml) was 1.7-fold higher ($P = 0.005$) than that in osteoarthritis synovial fluids (0.13 pmol/ml) (Fig. 1). Of note, the concentrations of adrenomedullin in Lyme arthritis samples are significantly different from that in osteoarthritis samples, even when the two highest Lyme arthritis points are not considered ($P = 0.012$). As a second point of reference, the concentration of adrenomedullin was measured in samples from rheumatoid arthritis patients, because rheumatoid arthritis is characterized by a more inflammatory phenotype than

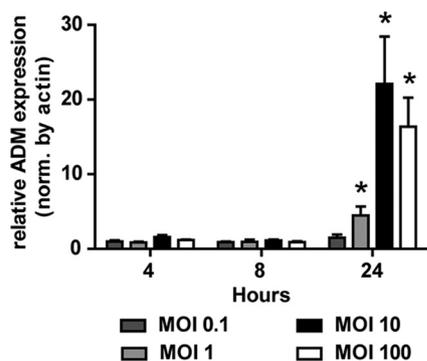


FIG. 2. *B. burgdorferi* coculture induces adrenomedullin expression in RAW264.7 cells. RAW264.7 cells were cocultured with *B. burgdorferi* for 4, 8, or 24 h. Adrenomedullin (ADM) expression was measured by qRT-PCR. The data shown are the mean induction of adrenomedullin compared to that of unstimulated samples (arbitrarily set to 1; data not shown), with error bars representing the standard error of the mean of three independent experiments. norm., normalized. *, $P = 0.037$.

Lyme arthritis. The median concentration of adrenomedullin in Lyme arthritis synovial fluids was 2.6-fold lower ($P = 0.005$) than that in rheumatoid arthritis synovial fluids (0.58 pmol/ml). Our data are consistent with previous reports suggesting that adrenomedullin production correlates with levels of inflammation and also demonstrate that adrenomedullin production is induced during Lyme arthritis.

***B. burgdorferi* coculture induces adrenomedullin expression in RAW264.7 cells.** In addition to inflammatory stimuli, adrenomedullin expression can be induced by bacteria and bacterial products (1, 35, 57, 59). To determine whether *B. burgdorferi* itself can induce the expression of adrenomedullin in host cells, RAW264.7 macrophages were cocultured with *B. burgdorferi* at various multiplicities of infection (MOI; 0.1, 1, 10, or 100), and adrenomedullin expression was measured by qRT-PCR. Adrenomedullin expression was significantly in-

duced in a dose-dependent manner after 24 h of coculture ($P = 0.037$) (Fig. 2).

The induction of adrenomedullin by *B. burgdorferi* requires signaling through MyD88, PI3-K, and p38. We next sought to identify which signaling molecules regulate adrenomedullin expression in our experimental system. We and others have previously shown that *B. burgdorferi* activates a number of signaling pathways, including Toll-like receptor 2 (TLR2)-MyD88 signaling and activation of the mitogen-activated protein kinases (MAPKs) p38, Jnk, and extracellular signal-regulated kinase (ERK), as well as activation of phosphatidylinositol 3-kinase (PI3-K) (4, 8, 21, 27, 48, 49, 54, 55). We therefore examined the potential roles for these molecules in the induction of adrenomedullin by *B. burgdorferi*.

To determine whether MyD88 is important for the regulation of adrenomedullin in response to *B. burgdorferi*, wild-type or MyD88^{-/-} bone marrow-derived macrophages were cocultured with *B. burgdorferi* at an MOI of 10 for 24 h. Adrenomedullin expression was measured by qRT-PCR. Deficiency in MyD88 resulted in an 83% decrease in adrenomedullin expression compared to the level in wild-type cells ($P = 0.014$) (Fig. 3A).

We next examined the potential roles in the induction of adrenomedullin for intracellular signaling molecules which are activated downstream of MyD88 in response to *B. burgdorferi*. RAW264.7 macrophages were pretreated with Ly294002 (10 μ M), which inhibits the activity of PI3-K and has been shown to inhibit the phagocytosis of *B. burgdorferi* (49), or with SB203580 (3 μ M), which inhibits the activity of p38 MAPK and the cellular response to *B. burgdorferi* (4, 8), prior to the addition of *B. burgdorferi* at an MOI of 10 for 24 h. Pretreatment with Ly294002 or SB203580 significantly inhibited the induction of adrenomedullin by 79% and 57%, respectively ($P = 0.037$) (Fig. 3B). Taken together, these data suggest that in response to *B. burgdorferi*, transmission of signals through the MyD88, PI3-K, and p38 MAPK pathways is necessary for the upregulation of adrenomedullin expression.

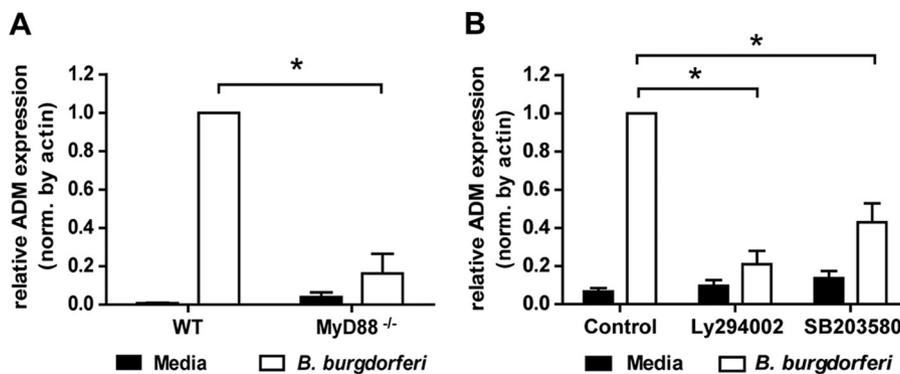


FIG. 3. The induction of adrenomedullin by *B. burgdorferi* requires signaling through MyD88, PI3-K, and p38. (A) Wild-type (WT) or MyD88-deficient (MyD88^{-/-}) bone marrow-derived macrophages were cocultured with *B. burgdorferi* at an MOI of 10 for 24 h. Adrenomedullin (ADM) expression was measured by qRT-PCR. The data shown are the mean expression of adrenomedullin relative to wild-type macrophages cocultured with *B. burgdorferi* (arbitrarily set to 1), with error bars representing the standard error of the mean of four independent experiments. *, $P = 0.014$. (B) RAW264.7 cells were pretreated with Ly294002 (10 μ M), SB203580 (3 μ M), or the control for 2 h prior to coculture with *B. burgdorferi* at an MOI of 10 for 24 h. Adrenomedullin expression was measured by qRT-PCR. Data shown are the mean expression of adrenomedullin relative to control cells cocultured with *B. burgdorferi* (arbitrarily set to 1), with error bars representing the standard error of the mean of three independent experiments. *, $P = 0.037$.

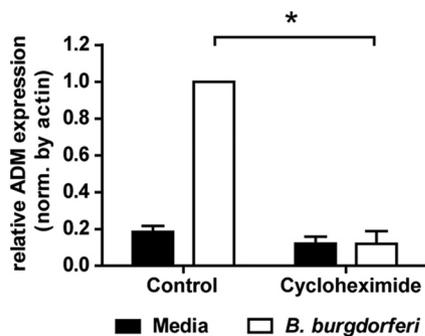


FIG. 4. The induction of adrenomedullin by *B. burgdorferi* requires new protein synthesis. RAW264.7 cells were cocultured with *B. burgdorferi* at an MOI of 10 for 24 h in the presence of cycloheximide (5 μ M) or the control. Adrenomedullin (ADM) expression was measured by qRT-PCR. The data shown are the mean expression of adrenomedullin relative to control cells cocultured with *B. burgdorferi* (arbitrarily set to 1), with error bars representing the standard error of the mean of four independent experiments. *, $P = 0.013$.

The induction of adrenomedullin by *B. burgdorferi* requires new protein synthesis. Previous work has demonstrated that adrenomedullin mRNA is induced in response to bacterial ligands as early as 2 h poststimulation (36, 59). The observation that *B. burgdorferi* coculture induces adrenomedullin mRNA only at later time points (Fig. 2) suggested that this induction may require synthesis of intermediary proteins rather than direct induction by recognition of *B. burgdorferi*. To determine whether protein synthesis is necessary for the induction of adrenomedullin mRNA, RAW264.7 macrophages were pretreated with cycloheximide (5 μ M), which inhibits mRNA translation on cytosolic 80S ribosomes, or the control for 2 h prior to the addition of *B. burgdorferi* at an MOI of 10 for 24 h. Pretreatment with cycloheximide significantly reduced (by 88%; $P = 0.013$) the induction of adrenomedullin mRNA in response to coculture with *B. burgdorferi* (Fig. 4). These results support the hypothesis that the inflammatory environment induced by *B. burgdorferi*, rather than *B. burgdorferi* itself, stimulates adrenomedullin expression.

Adrenomedullin decreases the *B. burgdorferi*-induced production of proinflammatory cytokines. Adrenomedullin has been shown to regulate inflammatory responses through the ligation of its receptor and the initiation of signaling cascades which downregulate the expression of proinflammatory mediators (25, 29, 41, 56). However, the immunomodulatory properties of adrenomedullin have never been examined in the context of an inflammatory response stimulated by a whole bacterium. We therefore sought to determine whether adrenomedullin can regulate the expression of inflammatory cytokines induced by *B. burgdorferi*.

RAW264.7 macrophages were cocultured with *B. burgdorferi* at an MOI of 1 for 4 h in the presence or absence of 100 nM adrenomedullin. This early time point is well before the time at which RAW264.7 cells begin expressing adrenomedullin in response to *B. burgdorferi* (Fig. 2). Thus, any affect of adrenomedullin can be attributed to the exogenous adrenomedullin added at the time of stimulation. The addition of adrenomedullin to *B. burgdorferi*-RAW264.7 macrophage cocultures did not decrease the expression of

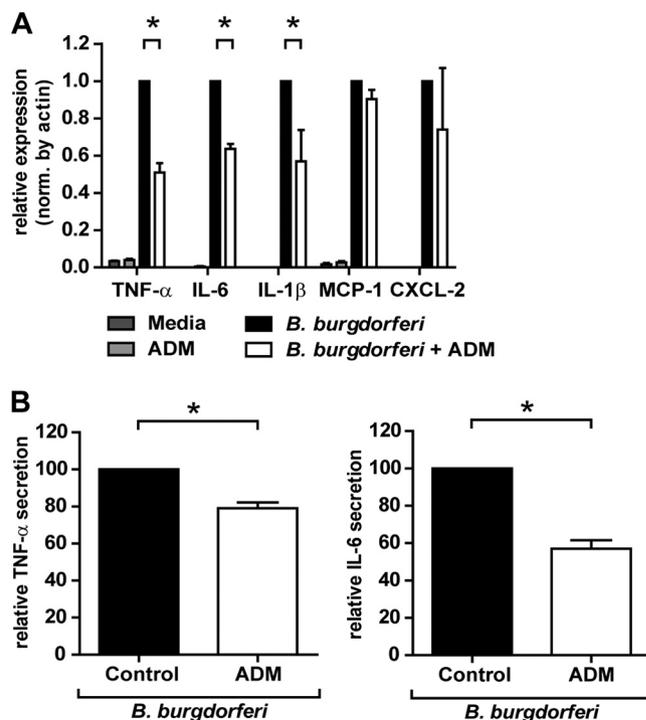


FIG. 5. Adrenomedullin decreases the *B. burgdorferi*-induced production of proinflammatory cytokines. (A) RAW264.7 cells were cocultured with *B. burgdorferi* at an MOI of 1 for 4 h in the presence of 100 nM adrenomedullin (ADM) or the control. Expression of inflammatory cytokines and chemokines was measured by qRT-PCR. The data shown are the mean expression of cytokines or chemokines relative to the level in control cells cocultured with *B. burgdorferi* (arbitrarily set to 1), with error bars representing the standard error of the mean of three independent experiments. *, $P = 0.037$. (B) RAW264.7 cells were cocultured with *B. burgdorferi* at an MOI of 10 for 6 h in the presence of 100 nM adrenomedullin or the control. Secretion of TNF- α and IL-6 was measured by ELISA. The data shown are the mean secretion of cytokines relative to control cells stimulated with *B. burgdorferi* (arbitrarily set to 100), with error bars representing the standard error of the mean of four independent experiments. Control cells secreted a mean of 29.16 ng/ml TNF- α , and adrenomedullin-treated cells secreted a mean of 23.028 ng/ml of TNF- α . Control cells secreted a mean of 0.825 ng/ml of IL-6, and adrenomedullin-treated cells secreted a mean of 0.470 ng/ml of IL-6. *, $P = 0.014$.

chemokines MCP-1 and CXCL-2 but did significantly decrease the expression of mRNA for inflammatory cytokines TNF- α (49%), IL-6 (36%), and IL-1 β (43%) ($P = 0.037$) (Fig. 5A).

To confirm this anti-inflammatory role of adrenomedullin, the concentrations of TNF- α and IL-6 in the supernatants of RAW264.7 cultures were measured by ELISA. IL-1 β is not secreted by RAW264.7 macrophages in response to *B. burgdorferi* (data not shown). RAW264.7 macrophages were cocultured with *B. burgdorferi* at an MOI of 10 for 6 h in the presence or absence of 100 nM adrenomedullin. In this experiment, a higher MOI and later time point were used to induce detectable levels of cytokine secretion. Similar to observations made with qRT-PCR, the addition of adrenomedullin to cocultures of RAW264.7 macrophages and *B. burgdorferi* significantly decreased the secretion of TNF- α and IL-6 by 21% and

43%, respectively (Fig. 5B). These data demonstrate that adrenomedullin is capable of regulating the host response to *B. burgdorferi* by reducing the induction of proinflammatory cytokines in macrophages.

DISCUSSION

Inflammation in response to infection is a double-edged sword for the host. While it is often a critical component in controlling bacterial burden, it can also cause as much or more damage than the infecting organism itself. As a result, there are multiple mechanisms by which the host dampens inflammation soon after initiation. It has previously been shown that, in addition to the activation of inflammatory pathways, *B. burgdorferi* also activates pathways releasing anti-inflammatory cytokines such as IL-4 and IL-10 (13, 17, 28, 31, 46, 50). The balance between the activation of inflammatory and anti-inflammatory pathways determines the phenotype of inflammation that is observed.

Here, we have presented our studies of an anti-inflammatory molecule, adrenomedullin, and its effects on *B. burgdorferi*-induced inflammation. Adrenomedullin expression is produced by cells such as lymphocytes and macrophages (35, 36, 42, 59) and modulates the inflammatory response both *in vitro* (25, 29, 41, 56) and *in vivo* (11, 18–20, 45, 52, 57, 58). The immunomodulatory effects of adrenomedullin involve the suppression of a TH1 proinflammatory response and the induction of anti-inflammatory cytokines and increase in regulatory T cells (18–20). We found that levels of adrenomedullin are increased in Lyme arthritis compared with levels in the less-inflammatory osteoarthritis, suggesting that *B. burgdorferi* infection results in the increased production of adrenomedullin. In addition, levels of adrenomedullin in Lyme arthritis are significantly lower than those seen in rheumatoid arthritis. Interestingly, there appeared to be some correlation of adrenomedullin levels with the typical severity of inflammation seen with the different forms of arthritis. Clinically, levels of inflammation with Lyme arthritis (as measured by leukocyte infiltration) are typically intermediate between those seen in rheumatoid arthritis and osteoarthritis, although there is considerable variability in the levels of inflammation seen in each condition. Our data are therefore consistent with previous reports that the production of adrenomedullin is upregulated under inflammatory conditions (22, 23, 36, 53). The correlation of adrenomedullin production with severity of arthritis suggests that, at a minimum, adrenomedullin is insufficient to completely control the inflammatory response. Whether inflammation in rheumatoid arthritis or Lyme arthritis would be more severe in the absence of adrenomedullin or whether additional adrenomedullin would be useful in further dampening inflammation remains unknown.

Using *in vitro* models, we have also shown that the addition of *B. burgdorferi* to RAW264.7 macrophages induces the expression of adrenomedullin in a dose-dependent manner. Adrenomedullin is induced *in vitro* upon coculture of RAW264.7 cells with *B. burgdorferi* in a dose-dependent manner. Previous work done by our laboratory and others has identified several major signaling pathways that are responsible for the regulation of the macrophage response to *B. burgdorferi*, including MyD88, PI3-K, and p38 (4, 7, 8, 27, 48, 49). MyD88 and PI3-K

are important for the phagocytosis of the spirochetes (49), and this phagocytosis is in turn crucial for the induction of proinflammatory cytokines (15, 48). In addition, we and others have demonstrated a role for p38 MAPK in the inflammatory response to *B. burgdorferi* (4, 8). Signaling through p38 is associated with Toll-like receptor-mediated responses and is involved in the upregulation of various proinflammatory molecules. We therefore hypothesized that inhibition of these molecules, and therefore known cellular responses to *B. burgdorferi*, may also inhibit subsequent adrenomedullin expression. Our data demonstrate that adrenomedullin expression in response to *B. burgdorferi* coculture is inhibited in MyD88^{-/-} macrophages, as well as by Ly294002 and SB203580, widely accepted inhibitors of PI3-K and p38, respectively. Taken together, our data suggest that the established mechanisms for the induction of the inflammatory response are also necessary for the later activation of adrenomedullin. This coregulation of pro- and anti-inflammatory processes may help to guarantee the control of the potentially deleterious inflammatory response. One caveat to these results is that neither Ly294002 nor SB203580 is completely specific for its primary target kinases, complicating interpretation of these data (5, 9, 32). Unfortunately, at this time, completely specific inhibitors of PI3-K and p38 are not commercially available.

The induction of adrenomedullin by *B. burgdorferi* occurs later than the first wave of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, which are increased within hours in response to bacterial ligands. In addition, in the case of TNF- α , induction often begins to decrease by as early as 4 to 6 h after exposure despite the ongoing presence of bacteria (16; M. L. Marre, unpublished data). The late induction of adrenomedullin after incubation of cells with *B. burgdorferi*, as well as the fact that an inhibitor of new protein synthesis reduces the induction of adrenomedullin mRNA, suggests that *B. burgdorferi*-induced inflammatory molecules, rather than *B. burgdorferi* itself, stimulate the expression of adrenomedullin. Indeed, previous reports have shown TNF- α to be sufficient for the induction of adrenomedullin expression (23, 36). Later induction of an anti-inflammatory molecule such as adrenomedullin by the early proinflammatory molecules would allow for the early development of inflammation, which may be required for control of infection. Then, after the initial burst of inflammation, induction of adrenomedullin and other anti-inflammatory molecules at later time points may serve to dampen inflammation and the associated tissue damage, allowing for more targeted control of inflammatory processes.

Many groups have demonstrated that adrenomedullin causes the downregulation of inflammatory cytokines in a target cell (25, 29, 41, 56). However, each of these experimental models involved the activation of cells through a single receptor, i.e., lipopolysaccharide (LPS) stimulating the production of inflammatory cytokines through Toll-like receptor 4 (56). Little is known about the ability of adrenomedullin to downregulate an inflammatory response induced by a whole bacterium that activates cells through numerous receptors. The addition of adrenomedullin to the medium of RAW264.7 cells cocultured with *B. burgdorferi* significantly decreased the production of inflammatory cytokines. These data suggest that the immunomodulatory activity of adrenomedullin is sufficient to

downregulate the expression of inflammatory mediators induced by whole *B. burgdorferi* cells.

The mechanisms by which adrenomedullin negatively regulates inflammatory responses have not been fully defined. Mature and active adrenomedullin transmits signals through a G protein-coupled receptor known as calcitonin receptor-like receptor (CRLR), which requires a receptor activity-modifying protein (RAMP) for transport to the plasma membrane (40). The signal transduction pathways downstream of this receptor are not well defined, but the ligation of CRLR by adrenomedullin results in an elevation in the level of the secondary messenger cyclic AMP (cAMP) within the cell as well as an increase in intracellular calcium concentrations (24, 47). The interaction of adrenomedullin with its receptor and subsequent signaling events ultimately results in tissue-specific changes in gene expression. Another potential mechanism by which adrenomedullin may affect bacterial infection is through its proposed effects as an antimicrobial agent. Adrenomedullin has been shown to inhibit the growth of an LPS-deficient strain of *Escherichia coli*, BUE55 (2). However, we were unable to find an effect of adrenomedullin on the growth of wild-type *E. coli* strains or on *B. burgdorferi* at concentrations of up to 1 μ M (M. L. Marre, unpublished data), making this a less likely mechanism for the role of adrenomedullin in our system.

The effect of adrenomedullin on inflammation *in vivo* remains unknown. The effect of exogenously administered adrenomedullin in dampening inflammation in a mouse model of rheumatoid arthritis has been reported (18). This model used a single intraperitoneal injection of rat adrenomedullin into mice that were then given intra-articular collagen injections to stimulate arthritis. However, similar experiments in a murine model of Lyme arthritis would be complicated by several caveats. Unlike the collagen-induced model of rheumatoid arthritis, murine Lyme arthritis develops over a period of 3 to 4 weeks, and treatment with a single dose of adrenomedullin is unlikely to be sufficient to control the inflammatory response over such a long span of time. Furthermore, the availability of only species-incompatible adrenomedullin limits the potential for repeated administration due to the development of a reaction to the foreign protein. An alternative to administering exogenous adrenomedullin to *B. burgdorferi*-infected mice would be to determine the effects of the absence of adrenomedullin in a knockout mouse model. However, deletion of the adrenomedullin gene results in embryonic lethality (10). These studies therefore await generation of conditional knockout mice which lack the expression of adrenomedullin in the joint tissue and monocytes.

In summary, we have shown that adrenomedullin is induced in response to *B. burgdorferi* both *in vivo* in the joints of infected patients and *in vitro* in macrophage cell cultures. We have also shown that adrenomedullin downregulates the inflammatory response to *B. burgdorferi* *in vitro*. These studies strongly implicate a role for adrenomedullin in the modulation of inflammatory responses associated with infection with *B. burgdorferi*. Future studies, perhaps utilizing conditional adrenomedullin knockout mice, will be needed to better understand the role of adrenomedullin at the various stages of infection with *B. burgdorferi* and to confirm its beneficial effect on Lyme arthritis.

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