Effect of *Borrelia burgdorferi* OspC at the Site of Inoculation in Mouse Skin[▽]

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The *Borrelia burgdorferi* surface lipoprotein OspC is a critical virulence factor, but its precise role in the establishment of *B. burgdorferi* infection remains unclear. To determine whether OspC affects the host response at the site of inoculation of the bacterium, the recruitment of macrophages and neutrophils and the production of cytokines were examined at the site of infection by wild-type, *ospC* mutant, and complemented mutant *B. burgdorferi* strains. Of the 21 cytokines tested, monocyte chemoattractant protein 1 (MCP-1), keratinocyte-derived chemokine (KC, CXCL1), and vascular endothelial growth factor (VEGF) were found at increased levels at the site of inoculation of *B. burgdorferi*, and the levels varied with the production of OspC at one or more time points over the 1-week course of infection. The kinetics of expression and the dependence on OspC production by *B. burgdorferi* varied among the cytokines. The production of KC and MCP-1, and the appearance of monocytic infiltrates, correlated with the presence of the bacteria rather than with OspC specifically. In contrast, VEGF production was not correlated simply to the presence of the bacteria and is influenced by the presence of OspC. In *in vitro* assays, OspC and *B. burgdorferi* expressing OspC stimulated the growth of endothelial cells more than did the controls. These data suggest the possibility of a novel role for OspC in the life of *B. burgdorferi* at the interface of its mammalian and tick hosts.

Lyme disease is the most common arthropod-transmitted infection in the United States and Europe (13), and the number of diagnosed cases is increasing every year in many locations (14). The disease is caused by the spirochete *Borrelia burgdorferi* (9), a bacterium with an unusual segmented genome and a life cycle requiring both an arthropod vector and a mammalian or avian host. Important traits of the bacteria that contribute to virulence have been revealed by *in vivo* and *in vitro* studies. One of the best-characterized virulence factors of *B. burgdorferi* is OspC, a surface-exposed lipoprotein that has been demonstrated to be essential for the colonization of the mammalian host in the tick-mouse infectious cycle (25, 61). Not only was OspC the first virulence determinant to be studied through the use of targeted genetic mutation and subsequent complementation in an infectious strain background (25, 49, 61), but it has also been the foundation for understanding the importance of the regulation of gene expression in *B. burgdorferi* (1, 11, 20, 30, 38, 56, 66).

The expression level of *ospC* is undetectable when *B. burgdorferi* is in unfed ticks but is increased dramatically as the tick feeds (55, 56). This dramatic change in gene expression led to an intensive examination of how *ospC* expression is regulated and led to the identification of the Rrp2-RpoN-RpoS regulatory cascade that governs the expression of a number of *B. burgdorferi* genes (11, 12, 30, 65, 66). While OspC expression is maximal at the time at which the bacteria are moving from tick

to mammal, expression wanes, and OspC-expressing bacteria are rapidly cleared as the adaptive immune response is activated (28, 36, 43, 63). Previous work investigated potential roles for OspC in overcoming challenges from the mammalian innate immune system (25, 54, 57) and in bacterial adaptation to the mammalian environment (57). Those studies, however, found that OspC is still required for infection of SCID and MyD88-deficient mice (25, 57), while *ospC* mutant bacteria are able to survive in dialysis membrane chambers implanted into the peritoneal cavity (57). Surprisingly, the role of OspC in mammalian infection can be partially replaced by the overexpression of other *B. burgdorferi* lipoproteins, although the mechanism of this phenomenon has not been investigated (62). Curiously, though, this work revealed a critical role for OspC in the colonization of some of the tissues examined, particularly in the heart, which is consistent with previously published results from two other laboratories (2, 28). A specific function for OspC in infection is supported by the recent demonstration that a site-directed change of one residue in a putative ligand-binding pocket rendered the bacteria noninfectious in the mouse model of infection (17). Despite intensive investigation, however, the role of OspC in *B. burgdorferi* infection remains unclear.

Although the role of OspC in the establishment of infection and its potential role in the interaction of the bacteria with the immune system have been studied *in vivo* (25, 36, 54, 57, 60, 61, 63), the role of OspC in the interaction of the bacteria with the innate immune system specifically at the site of inoculation in the skin has not been reported. In this work, we investigated the possibility that OspC may affect the response of macrophages cultured *in vitro* to *B. burgdorferi* and then moved on to examine the role of OspC at the site of inoculation in mouse skin, which cannot be recapitulated *in vitro*. Because of the

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TABLE 1. *Borrelia burgdorferi* strains used in this study

<i>Borrelia burgdorferi</i> strain	Genotype	Antibiotic resistance(s)	Reference
B31 A3	WT (all plasmids except cp9)	None	22
B31 A3 OspCK1 (Δ C)	$\Delta ospC::flgBp-kan$	Kanamycin	61
B31 A3 OspCK1/pBSV2G-OspC (Δ C/pOspC)	$\Delta ospC::flgBp-kan$ + pBSV2G- <i>ospC</i> ; <i>ospC</i> under the control of the native promoter	Kanamycin, gentamicin	61

important role that cytokines can play in the outcome of infection, we examined the production of various cytokines *in vivo* after infection with *Borrelia burgdorferi* strains that do and do not express OspC.

MATERIALS AND METHODS

Mouse and bacterial strains. Female 6- to 8-week-old C57BL/6J and C3H/HeN mice were obtained from commercial sources and housed in the facilities of the Tufts-New England Medical Center Division of Laboratory Animal Medicine. All procedures used were reviewed and approved by the Tufts University Institutional Animal Care and Use Committee.

The bacterial strains used for all work reported here are described in Table 1. The bacteria were grown in Barbour-Stoenner-Kelly (BSK) II medium (5) at 32°C. Growth was monitored by dark-field microscopy, and once the bacteria reached a density of 1×10^7 to 5×10^7 cells/ml, they were frozen in single-experiment aliquots in BSK II medium containing 20% glycerol. To confirm that OspC was appropriately expressed by the different strains, a Western blot was performed on bacterial lysates using a polyclonal rabbit antiserum directed against OspC (a generous gift from Tom Schwan, Rocky Mountain Laboratories, Hamilton, MT).

***In vitro* infection of macrophages with *B. burgdorferi*.** Primary cultures of bone marrow-derived macrophages (BMM ϕ s) were prepared and used for the *in vitro* infections of macrophages with *B. burgdorferi* essentially as previously described (15, 58). The marrow of four femurs (two mice) per mouse strain was pooled for each experiment. The cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10% fetal bovine serum (FBS), and 30% L cell-conditioned medium. The bone marrow cells were plated onto untreated plastic dishes and fed on day 3 by the addition of 10 ml of fresh medium. On day 5, the nonadherent cells were removed, and the adherent cells were replated into six-well tissue culture plates at a density of 5×10^5 cells/well. Prior to infection on day 7, the medium was removed, the wells were washed with phosphate-buffered saline (PBS) to remove antibiotics, and the bacteria (B31 A3 wild type [WT], Δ C, and Δ C/pOspC) were added at a multiplicity of infection (MOI) of 10:1 or 100:1 in 1 ml of BMM ϕ medium without antibiotics. Control wells were incubated with medium alone. The plates were centrifuged at $200 \times g$ for 15 min at room temperature to synchronize infection and maximize bacterium-macrophage contact. The plates were incubated at 37°C in an atmosphere of 5% CO₂ for 3 h. After infection the supernatants from each well were collected, filtered through a 0.22- μ m filter, and stored at -80°C for subsequent analysis of cytokine secretion. The cells were harvested for analysis of mRNA.

***In vitro* infection of endothelial cells with *B. burgdorferi*.** The Ea.hy926 endothelial cell line (18, 19) was plated onto Matrigel (BD Biosciences, San Jose, CA) or onto conventional tissue culture-treated plastic in standard growth medium (Dulbecco's modified Eagle medium with high glucose, 10% heat-inactivated fetal bovine serum [Gibco, Grand Island, NY], hypoxanthine-aminopterin-thymidine [HAT] medium supplement [Sigma-Aldrich, St. Louis, MO], and 2 mM L-glutamine). Two days after plating, the cells were treated with purified vascular endothelial growth factor (VEGF) (obtained from the NCI repository) or infected with *B. burgdorferi* at an MOI of 10:1. The growth of the endothelial cells was monitored and structure formation was quantified visually for 7 to 10 days, depending on the lot of Matrigel. Data were analyzed for statistical significance by using one-way repeated-measures analysis of variance (ANOVA) and Dunnett's posttest. Supernatants from *B. burgdorferi*-infected wells were taken for analysis of VEGF levels by an enzyme-linked immunosorbent assay (ELISA) using the Quantikine human VEGF immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. In other experiments, the Ea.hy926 cells were plated in growth medium without antibiotics onto 3- μ m-pore-size transwell inserts, allowed to reach confluence over 2 to 3 days, and then infected with *B. burgdorferi* at an MOI of 20 and monitored over 72 h. Bacteria

that had crossed the membrane or remained in the inserts were enumerated, and data were analyzed by using one-way repeated-measures ANOVA followed by Bonferroni's multiple-comparison test.

In similar experiments, purified recombinant proteins were also tested for effects on endothelial cell growth and structure formation. Purified maltose-binding protein (MBP)- β -galactosidase (β gal) and MBP-OspC were described previously (2). Purified proteins were tested at 1 and 10 μ g/ml. The synthetic lipopeptide Pam3CSK4 (InvivoGen, San Diego, CA) was used at the same concentrations in some experiments.

Infections of C3H/HeN mice with *B. burgdorferi*. To infect mice, frozen aliquots of bacteria were thawed, washed, and cultured overnight at 32°C in BSK II medium. The bacteria were enumerated by using a Petroff-Hausser counting chamber under dark-field microscopy and were suspended in BSK II medium to 1.25×10^8 bacteria/ml. C3H/HeN mice were inoculated intradermally in the dorsal thoracic area with 5×10^3 motile spirochetes in 40 μ l of BSK II medium or were mock infected with BSK II medium alone. To confirm the number of spirochetes injected into the mice, aliquots from each sample were plated onto BSK II plates, and the colonies were enumerated. The injection sites were labeled with a marker immediately after inoculation to ensure that the samples would be collected from that site. The inoculation of cultured bacteria, as opposed to tick-mediated delivery, was used to ensure that equal numbers of bacterial cells were introduced into all animals. Mice were euthanized 6 h, 1 day, 2 days, and 7 days later, and skin samples were collected at the inoculation site, divided into three equal parts, and snap-frozen in RNase- and DNase-free preweighed microcentrifuge tubes.

Cytokine extraction from skin and analysis of cytokines by Luminex. Cytokines were extracted from skin samples by using a slight modification of a protocol described previously (41). Briefly, the samples were weighed, and 1 ml of ice-cold cytokine extraction buffer was added. The buffer consisted of PBS; 0.1% Igepal CA-630, a nonionic detergent; and three protease inhibitors (0.01 trypsin inhibitory units [TIU] per ml aprotinin, 1 mM benzamide, 1 μ g/ml leupeptin). The samples were homogenized with a tissue disrupter (Omni shredder model TH-115; USA Scientific). The homogenates were centrifuged for 5 min at $9,300 \times g$ at 4°C, and the supernatants were collected and stored at -80°C for cytokine analysis. Multianalyte profiling (xMAP) technology (Luminex, Austin, TX) was used for the simultaneous quantification of various mouse cytokines and chemokines in both the tissue extracts and the supernatants harvested from macrophages in culture. The 21-plex mouse cytokine bead kit was obtained from Millipore (Billerica, MA). The detection limit (or assay sensitivity) was 3.2 pg/ml. The analysis was performed by the Clinical and Translational Research Center at Tufts Medical Center. Statistical significance was assessed by the nonparametric Mann-Whitney test using GraphPad Prism software. The significance level was set to a *P* value of <0.05. The *P* value is indicated in the figures when less than 0.05.

RNA preparation and reverse transcription (RT)-PCR analysis. For the *in vitro* BMM ϕ infections, total RNA was isolated by using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The samples were then treated with the DNA-free kit from Ambion (Austin, TX) as described by the manufacturer. The DNA-free total RNA was reverse transcribed into cDNA in a final volume of 20 μ l using 500 ng of RNA, 5 μ M random hexamers, 10 mM deoxynucleoside triphosphate (dNTP) mix, and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR using SYBR green PCR master mix (Qiagen) was used to determine the expression levels of the cytokines in the samples. All reactions were performed for 40 cycles in a final volume of 25 μ l in 96-well plates with a Stratagene (La Jolla, CA) MX3000p real-time thermocycler, and the data were analyzed by using the accompanying software. For the quantification of the cytokines, primers listed in Table 2 were used, and the transcript levels are presented as the fold difference over WT-infected samples after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values for the same samples.

For the mouse skin samples, the tissues were homogenized by using an Omni shredder (model TH-115) in buffer RLT, provided with the RNeasy kit (Qiagen),

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence	Reference
MCP-1F	GCTGTTACAGTTGCCGGCT	3
MCP-1R	CATTAGCTTCAGATTTACGG	3
KC-F	GAGAGAATGGTCGCGAGGCTTG	This study
KC-R	CTCTTTCTTTTCGCACAACACCC	This study
IL-10F ^a	GTGTGGTTGCCAAGCCTTAT	This study
	CGGA	
IL-10R ^a	ACCTACCTGCTCCACTGCCTTGCT	This study
TNF- α F ^a	CTCTTTCTGTCTACTGAACCTCGG	This study
	GGTGATCGGTCC	
TNF- α R ^a	GTGTGTATGAGATAGCAAATCGG	This study
	CTGACGGTGTGGG	
actinF	TCAACACCCAGCCATGTAC	This study
actinR	TTTCCAGCCTTCCTTCTT	This study
nidoF	CACCCAGCTTCGGCTCAGTA	32
nidoR	TCCCCAGGCCATCGGT	32
flaF	TCTTTTCTCTGGTGAGGGAGCT	32
flaR	TCCTTCCTGTTGAACACCCTCT	32

^a The final MgCl₂ concentration was 2.5 mM for these primers. All others had no additional MgCl₂ added and were used in the reaction buffers from the kits. The annealing temperature used for all reactions was 60°C.

and total RNA was isolated by using the RNeasy kit according to the manufacturer's instructions (Qiagen). The samples were DNase treated on column by using the RNase-free DNase set from Qiagen as described by the manufacturer. DNase-free total RNA was then reverse transcribed, as described above. The values are presented as copies of the cytokine normalized to mouse β -actin mRNA levels in the same samples. To allow the quantification of the copy number in each sample, a standard curve was created, which consisted of known quantities of recombinant plasmid DNA specific for each gene tested.

DNA isolation and quantitative PCR. Skin samples were homogenized in 300 μ l of buffer ATL, provided with the DNeasy tissue kit (Qiagen), with an Omni shredder (model TH-115), and the DNA was extracted from the tissues by using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. To obtain RNA-free DNA samples, RNase A (Roche, Basel, Switzerland) was added to the samples during the purification process prior to the passage of the homogenates through the Qiagen columns. To determine the spirochete load, real-time PCRs for *B. burgdorferi* flagellin (*flaB*) and for the mouse nidogen (*nido*) gene were performed by using the primers described in Table 2. The reactions were performed by using SYBR green PCR master mix (Qiagen) for 40 cycles in a final volume of 25 μ l in 96-well plates with a Stratagene MX3000p real-time thermocycler, and the data were analyzed by using the accompanying software. Standards and samples were simultaneously amplified in the same plates. The *flaB* copy number standard contained purified *B. burgdorferi* DNA mixed with DNA isolated from uninfected mouse tissue. For the *nido* standard curve, purified mouse DNA was used.

Skin histopathology. Skin samples from the mouse infections described above were collected and fixed in 2 ml of formalin. The samples were embedded in paraffin, and skin sections were stained with hematoxylin and eosin (H&E) by the Histology Core Facility at Tufts University. Lauren Richey, an independent pathologist who was blinded to the experimental design, scored the sections. For neutrophils, the score was on a scale of 0 to 4, where 0 was no neutrophils per high-power field (hpf), 1 was 1 neutrophil per hpf, 2 was 2 to 5 neutrophils per hpf, 3 was 6 to 20 neutrophils per hpf, and 4 was 21 neutrophils and above per hpf. The presence of monocytes was scored on a scale of 0 to 4, where 0 was no infiltrates (no notable mononuclear cell populations), 1 was a slight scatter of cells (small number of scattered mononuclear cells), 2 was mild infiltrates, 3 was moderate infiltrates, and 4 was dense infiltrates (infiltrates of mononuclear cells arranged in clumps or sheets). The presence or formation of new capillaries or arterioles over time (the vascularization score) was on a scale of 0 to 4, where 0 indicated that subcutaneous capillaries are inconspicuous; 1 indicated occasional small capillary-sized blood vessels; 2 indicated occasional small capillary-to-arteriole-sized vessels; 3 indicated frequent small-to-arteriole-sized vessels, 1 to 5 red blood cells (RBCs) wide; and 4 indicated many large dilated vessels. Statistical significance was assessed by one-way ANOVA. The significance level was set at a *P* value of <0.05. The *P* value is indicated whenever it was less than 0.05.

Statistical analyses. Statistical analyses of all quantitative data were performed by using GraphPad (GraphPad Software, Inc., San Diego, CA). Sta-

tistically significant differences between samples are indicated in the figures and/or legends. If comparisons are not indicated as statistically significant, they were not.

RESULTS

OspC does not affect expression and secretion of cytokines by murine macrophages *in vitro* after infection by *Borrelia burgdorferi*. Macrophages are the cells that first respond to the site of *Borrelia burgdorferi* infection (42), and there have been extensive studies on macrophage infections with *Borrelia burgdorferi in vitro* (15, 23, 24, 35, 39, 42, 44, 46, 53). The hypothesis that OspC might play a role in the interaction of the bacteria with the innate immune response led to the investigation of the interaction of macrophages with WT and *ospC* mutant bacteria. To address this question, the cytokine responses of macrophages from two different mouse strains infected with *B. burgdorferi in vitro* were tested at the mRNA and the protein levels.

Primary cultures of bone marrow-derived macrophages were infected with WT B31 A3, the *ospC* mutant (Δ C), and the complemented mutant (Δ C/pOspC) at an MOI of 10:1 or 100:1 for 3 h. Total RNA was harvested from the cells and was used to quantify the levels of tumor necrosis factor alpha (TNF- α), interleukin-10 (IL-10), keratinocyte-derived chemokine (KC, CXCL1), and monocyte chemoattractant protein 1 (MCP-1) mRNA. These cytokines were chosen because they are known to be secreted during infections of macrophages with *Borrelia burgdorferi* and have proinflammatory (TNF- α , KC, and MCP-1) and anti-inflammatory (IL-10) actions (4, 8, 15, 24, 27, 35, 46, 53). To determine whether any observed differences in RNA transcript levels correspond to differences in cytokine production, the supernatants from each well were also analyzed for cytokine content.

The levels of cytokine mRNAs were normalized to that of the housekeeping gene GAPDH and are expressed as a fold difference compared to those induced in response to infection by WT *B. burgdorferi* strain B31 A3. The expression levels of the cytokine mRNAs were not affected by the expression of OspC by *B. burgdorferi* (Fig. 1), as there were no statistically significant differences in the levels produced by macrophages infected with the WT, Δ C, and Δ C/pOspC *B. burgdorferi* strains. The levels of cytokine proteins in the supernatants reflect the expression levels of the mRNAs, with no significant difference between cells infected with the three *B. burgdorferi* strains in any comparison. The only difference detected in the mouse strain-dependent responses to *B. burgdorferi* was that IL-10 was not detected in the supernatants of the C3H macrophages at any MOI tested but was detected in macrophages from C57BL/6 mice (data not shown). The absence of an OspC-dependent effect on the secretion of proinflammatory or anti-inflammatory cytokines by macrophages in culture is consistent with the observation that there was no difference in the numbers of cell-associated bacteria between the different *B. burgdorferi* or mouse strains (data not shown), suggesting that OspC is not essential for the attachment or uptake of the bacteria. Under all conditions tested, macrophage viability was unaffected by the expression of OspC by *B. burgdorferi* (data not shown).

Infection of C3H/HeN mice with the B31 A3 WT, Δ C, and Δ C/pOspC strains. To determine the response of the innate

immune system to *B. burgdorferi* strains that do or do not express OspC at the site of infection, C3H/HeN mice were inoculated intradermally in the dorsal thoracic area with 5×10^3 bacteria. Again, the strains tested were wild-type (WT) B31 A3, the OspC mutant (ΔC), and the complemented mutant ($\Delta C/pOspC$). The dose used has been shown in previous work to ensure infection if OspC is present. As a control for the inoculation process, mice were injected with the same volume of BSK II medium. At 6 h, 1 day, 2 days, and 7 days after inoculation, the mice were euthanized, and skin samples were collected from the site of the injection. These time points were chosen because previous work showed that bacteria that do not express OspC are not recovered from the site of infection after 2 days postinfection (61). The skin samples were homogenized and the cytokines were extracted from the tissues as described in Materials and Methods, based on a previously reported protocol (41). The levels of 21 cytokines were quantified by using Luminex bead technology. Ten cytokines were present at detectable levels (IL-1 α , IL-2, IL-6, IL-10, IL-12p40, RANTES, gamma interferon [IFN- γ], MCP-1, KC, and VEGF), but only MCP-1, KC, and VEGF showed a statistically significant difference between the uninfected and the WT-infected samples at any time point (data not shown). These cytokines were chosen for further analyses.

Subsequent *in vivo* experiments were performed as described above except that the skin samples from the site of inoculation were split into three equal parts. One part was fixed and examined at the histological level by a pathologist, and the other parts were used for cytokine extraction and nucleic acid extraction. The levels of the MCP-1, KC, and VEGF proteins were quantified in parallel with the corresponding RNA levels, and all data were analyzed for statistical significance. Figure 2 shows the levels of cytokine mRNA and protein levels in the skin at the site of inoculation over the time course of infection. At 6 h postinfection there was a statistically significant difference between the WT-infected and the uninfected samples ($P < 0.05$) in the production of the MCP-1 protein. At 1 day MCP-1 could be detected in all samples, but the levels of the cytokine had declined, and there was no

FIG. 1. Expression and secretion of TNF- α , IL-10, KC, and MCP-1 after *in vitro* infection of murine macrophages by *B. burgdorferi*. mRNA levels for the cytokines TNF- α , KC, and MCP-1 were quantified by RT-PCR after 3 h of infection of bone marrow-derived macrophages from C3H/HeN mice at an MOI of 10:1. The data were normalized to the housekeeping gene GAPDH and are expressed as the fold difference compared to infection by the B31 A3 WT strain. The supernatants from the infections were collected and assayed by using Luminex technology for the secretion of the same cytokines. Shown are the means of all replicates from at least two independent determinations $\pm$ the standard errors; macrophages from C57BL/6 mice were similar, as were results from infections performed at an MOI of 100. At the mRNA level, statistically significant differences were as follows: a P value of <0.05 for WT- and ΔC -infected versus uninfected mice for TNF- α and KC and a P value of <0.05 between WT-infected and uninfected macrophages for MCP-1, but there were no significant differences between infections by the different *B. burgdorferi* strains. At the protein level, there were no statistically significant differences between infections with any of the three *B. burgdorferi* strains for any cytokine, but for KC and TNF- α there were statistically significant differences ($P < 0.05$) between the infected and uninfected samples.

FIG. 2. Expression and production of MCP-1, KC, and VEGF at various time points in skin of infected mice. The expression and production of cytokines in the skin samples were assessed by RT-PCR and Luminex technology, respectively. For MCP-1, mRNA was reproducibly detected in WT and $\Delta C/pOspC$ samples at 7 days postinfection. At 6 h there was a statistically significant difference in the levels of MCP-1 between the WT-infected and the uninfected samples ($P < 0.05$). At 1 day the levels of MCP-1 dropped, and at 2 days the levels of MCP-1 were below the limit of detection (not shown). At 7 days postinfection MCP-1 was detected in WT- and $\Delta C/pOspC$ -infected skin, and these samples were statistically significantly different compared to the uninfected samples ($P < 0.001$ for both strains) and the ΔC mutant-infected samples ($P < 0.001$ and $P < 0.05$, respectively). In the same samples, KC was detected at 6 h and 1 day but was below the limit of detection at 2 days (not shown). At 7 days KC was detectable in the samples from mice infected with all three *B. burgdorferi* strains, but the levels were statistically significantly higher than those of the uninfected controls only for the WT- and the $\Delta C/pOspC$ -infected samples ($P < 0.01$ for both). At 6 h and 1 day postinfection, there was no statistically significant difference between the samples. VEGF was detectable in response to the presence of bacteria at 6 h and 1 day but was below the limit of detection at 2 and 7 days. The production of VEGF at 1 day postinfection is statistically significant for the WT-, $\Delta C/pOspC$ ($P < 0.01$), and ΔC ($P < 0.05$)-infected samples compared to the uninfected controls. There is not a statistically significant difference between the WT- and ΔC - or $\Delta C/pOspC$ - and ΔC -infected samples. For all panels, the bars represent the geometric means; the open symbols represent samples that were below the limit of detection of the assay, and statistical analyses were performed. A single asterisk denotes a P value of < 0.05 , and a double asterisk denotes a P value of < 0.01 by the Mann-Whitney test.

statistical significance between any samples. Correspondingly, the mRNA levels also declined sharply. At 2 days the levels of MCP-1 were below the limit of detection in all samples (data not shown). At 7 days postinfection MCP-1 was present in WT-

and $\Delta C/pOspC$ -infected mouse skin at levels that were significantly different from those present in the uninfected ($P < 0.001$) or the ΔC -infected samples ($P < 0.001$ and $P < 0.05$). Aside from the above-mentioned differences, there were no

statistically significant differences in cytokine mRNA or protein levels in skin samples from mice infected with the three bacterial strains. Comparable results were obtained when protein levels were measured in the same samples by ELISA (data not shown). These results support previously reported data from other groups showing that MCP-1 is produced during infection by *Borrelia burgdorferi* (26, 67), but this is the first report that infection with WT *Borrelia burgdorferi* induces the production of MCP-1 in the skin at the site of the infection. The levels of expression of MCP-1 mRNA were also assessed in the same samples, and the values reflect the trends at the protein level.

The levels of KC at the protein and RNA levels in the skin at the site of inoculation are also presented in Fig. 2. There is secretion of KC in response to the presence of bacteria at 6 h and 1 day, although there were no significant differences from the uninfected controls. At 2 days neither protein nor mRNA was detected (data not shown). At 7 days the production of KC reappeared, similar to what was seen for MCP-1. The higher levels of KC in the WT and Δ C/pOspC samples than in the uninfected samples at 7 days postinfection are statistically significant ($P < 0.01$). The results support data from other groups showing that KC is produced due to *Borrelia burgdorferi* infection (7, 10, 64), but again, this is the first time that *Borrelia burgdorferi* infection is shown to induce the production of KC in the skin at the site of infection. The levels of expression of mRNA were also assessed in the same samples, but KC mRNA has a very short half-life, which may account for the absence of detectable levels of KC mRNA in most of the samples.

Figure 2 also shows the data for the VEGF protein in the skin at the site of inoculation. The production of VEGF at 1 day postinfection was statistically significantly different from the those of uninfected controls for the WT ($P < 0.01$), Δ C ($P < 0.05$), and Δ C/pOspC ($P < 0.01$) infections. There was not a statistically significant difference between the infection samples for the WT and Δ C or for Δ C/pOspC and Δ C strains, but there was a trend toward lower levels in the samples taken from mice infected with the Δ C mutant. This is the first report of VEGF production in response to *Borrelia burgdorferi* infection at the site of inoculation in mice, but a recent publication by Myers et al. documented VEGF production in an *in vitro* culture system in which mechanisms of damage to neurons were investigated (47).

Spirochete loads at various time points. The cytokine levels were detectable initially and declined, and the levels of KC and MCP-1 then rebounded. To determine whether this pattern reflects bacterial numbers, the bacterial loads were determined by quantitative PCR (QPCR) (Fig. 3). There were bacteria detectable at 6 h postinfection, but their numbers decreased by 1 day postinfection. The numbers of the Δ C bacteria at 1 day postinfection were higher than those of the WT and Δ C/pOspC strains, suggesting a role for OspC in facilitating dissemination from the site of inoculation. At 7 days postinfection the bacterial numbers were higher than at other time points tested for the WT- and Δ C/pOspC-infected mice, but the Δ C bacteria were undetectable, confirming previous reports that the Δ C bacteria were not detected after 2 day postinfection (61). These results show that detectable bacterial genomes correlate with detectable levels of MCP-1 and KC. In contrast, the VEGF response was seen exclusively at the earliest stage of

FIG. 3. Spirochete loads at various time points. Bacterial loads at the inoculation site were determined by QPCR using primers listed in Table 2 and are expressed as flagellin (*flaB*) copies per 10,000 nidogen (*nido*) copies. The limit of detection was 160 bacteria. There were bacteria present at 6 h postinfection, but their numbers decreased and were below the limit of detection at 1 day postinfection. At 7 days postinfection the numbers of bacteria increased for the WT- and Δ C/pOspC-infected samples ($P < 0.01$ compared to the uninfected controls and the mice infected with Δ C; this was the only statistically significant difference between infection groups within the same time point). The bars represent the geometric means, and the open symbols represent samples that were below the limit of detection. The y axis (bacterial load) is presented in three segments due to the drastically different numbers of bacteria present in the skin at the inoculation site at different times.

infection and does not simply reflect bacterial burdens in the skin, as VEGF was not detected at 7 days postinfection.

Histopathological analyses of skin from the site of *B. burgdorferi* infection *in vivo*. To determine how effector cells of the innate immune system respond to infection with the WT, the Δ C mutant, and the Δ C/pOspC complemented mutant, the infiltration of immune cells was assessed at the site of the infection at 6 h, 1 day, 2 days, and 7 days by histopathology. Skin sections were H&E stained, and an independent pathologist who was blinded to the experimental design scored them for the presence of neutrophils and monocytes and the presence or formation of new capillaries or arterioles over time, which is the vascularization score. The results are presented in Table 3. There was no statistically significant difference in the numbers of neutrophils or in vascularization at any time point under any infection condition.

In contrast, the infiltration of monocytes in response to injection was seen by 1 day, and the numbers of monocytes were statistically significantly higher in the mice infected with the WT than in the uninfected and the Δ C samples at 7 days postinfection. These results show that after infection with *Borrelia burgdorferi*, the inflammatory response consists predominantly of the recruitment of monocytes to the site of infection. This inflammatory response in the skin was described previously by other groups as well (29, 64). The presence of monocytes at 7 days postinfection in WT and Δ C/pOspC samples is consistent with the data showing a production of MCP-1 and KC, and with the bacterial burdens, at the same time point.

Potential intersection of *B. burgdorferi* and VEGF-mediated events. While the absence of OspC does not significantly affect the initial recruitment of innate immune cells to the site of the infection in the skin or the initial levels of the

TABLE 3. Histopathology scoring for the presence of mononuclear cells, neutrophils, and vascularization in mouse skin at the site of inoculation of *B. burgdorferi*

Histopathology tested and infection group ^a	Avg histopathology score $\pm$ SD			
	6 h	1 day	2 days	7 days
Mononuclear cells				
Uninfected	1.8 $\pm$ 0.6	2.3 $\pm$ 0.8	2.1 $\pm$ 0.7	1.2 $\pm$ 0.7
WT	1.9 $\pm$ 0.3	2.8 $\pm$ 0.6	2.2 $\pm$ 0.6	2.5 $\pm$ 0.6 ^b
Δ C	2.1 $\pm$ 0.3	2.2 $\pm$ 0.6	2.0 $\pm$ 0.9	1.5 $\pm$ 0.6
Δ C/pOspC	1.8 $\pm$ 0.6	2.1 $\pm$ 0.5	2.2 $\pm$ 0.6	2.1 $\pm$ 0.6
Neutrophils				
Uninfected	2.8 $\pm$ 0.9	2.3 $\pm$ 0.9	2.1 $\pm$ 0.8	1.5 $\pm$ 0.9
WT	3.6 $\pm$ 0.5	2.2 $\pm$ 0.7	2.2 $\pm$ 0.8	2.3 $\pm$ 1.1
Δ C	2.6 $\pm$ 0.9	2.3 $\pm$ 0.7	1.5 $\pm$ 0.6	1.5 $\pm$ 0.5
Δ C/pOspC	3.3 $\pm$ 0.8	2.2 $\pm$ 0.8	1.9 $\pm$ 0.6	1.7 $\pm$ 0.8
Vascularization^c				
Uninfected	1.6 $\pm$ 0.6	1.6 $\pm$ 0.9	1.9 $\pm$ 0.7	1.2 $\pm$ 1.1
WT	2.1 $\pm$ 0.5	2.0 $\pm$ 0.9	2.0 $\pm$ 0.7	1.5 $\pm$ 0.9
Δ C	1.8 $\pm$ 1.0	1.9 $\pm$ 1.0	1.7 $\pm$ 0.6	1.3 $\pm$ 0.8
Δ C/pOspC	2.3 $\pm$ 0.4	1.9 $\pm$ 0.9	1.9 $\pm$ 0.7	1.5 $\pm$ 0.9

^a Each group consisted of 10 to 14 mice.

^b $P = 0.01$ in comparison to uninfected and Δ C-infected samples. No other comparisons in the histopathology scores showed statistically significant differences.

^c Vascularization was scored in parallel with other assessments, although no changes in the numbers of vessels would necessarily be expected.

chemokine MCP-1 or KC, the early increase in VEGF levels was an intriguing result in the response to an arthropod-transmitted pathogen. Although the acronym stands for vascular endothelial growth factor, VEGF has another role, i.e., inducing the permeability of vessels, and was originally known as vascular permeability factor. In fact, the transient increase in the level of VEGF is more likely to affect vascular permeability than the appearance of new vessels, which is consistent with our histopathological results. The production of VEGF in response to infection by *Borrelia burgdorferi* could lead to increased vascular permeability, which may facilitate bacterial exit from the site of inoculation and dissemination to distant sites. To determine whether *B. burgdorferi* can mimic VEGF effects, or induce VEGF secretion that has downstream effects on endothelial cells, we used *in vitro* cell culture models.

First, to determine whether OspC affects the ability of *B. burgdorferi* to cross endothelial cell monolayers *in vitro*, Ea.hy926 endothelial cells were plated into transwell inserts and grown to confluence. Inserts without endothelial cells served as positive controls to ensure that bacterial motility would result in countable numbers of bacteria crossing the membrane. Bacteria were then added at an MOI of 20, and samples were taken over the next 72 h from both the insert and the well (containing bacteria that had crossed the cell layers or control membranes) for the enumeration of the bacteria. There were no significant differences between the WT, Δ C, and Δ C/pOspC *B. burgdorferi* strains in their ability to transmigrate across Ea.hy926 endothelial layers (Fig. 4).

In a second approach to determine whether *B. burgdorferi*, and OspC in particular, affects endothelial cells, Ea.hy926 cells were plated onto Matrigel and then infected with *B. burgdorferi* or treated with VEGF. In this system, endothe-

lial cells treated with VEGF expand more rapidly than the untreated controls, and they form multicellular structures (33, 40). Consistent with those previous reports, we observed a more rapid growth of the cells treated with VEGF and, to our knowledge, for the first time, of the cells infected with *B. burgdorferi* (Fig. 5). In comparisons of *B. burgdorferi* strains that do and do not express OspC in this system, the Δ C mutant bacteria did promote endothelial cell growth and structure formation and in some experiments were signifi-

FIG. 4. OspC does not affect the ability of *B. burgdorferi* to cross endothelial layers *in vitro*. Ea.hy926 endothelial cells were plated in the absence of antibiotics in 3- μ m-pore-size transwell inserts and grown to confluence. Inserts without cells served as controls. Bacteria were then added at an MOI of 20, and samples were taken over the next 72 h from both the insert and the well (containing bacteria that had transmigrated across the cell layers or membranes). The bacteria were enumerated by using a Petroff-Hausser counting chamber. The total numbers of bacteria were then calculated, as the volumes in the insert are different from those in the well, and the percentage of the total bacteria in each well was calculated. Data were analyzed for statistical significance by one-way repeated-measures ANOVA followed by Bonferroni's multiple-comparison test. Shown are the means plus standard deviations of data from three independent experiments. For each of the three *B. burgdorferi* strains tested (WT, Δ C, and Δ C/pOspC), there was a significant difference in migration in comparisons of wells containing endothelial cells versus no cells ($P < 0.001$), but there were no significant differences in comparisons of the three bacterial strains ($P > 0.05$).

FIG. 5. Effects of VEGF, *B. burgdorferi*, and OspC on endothelial cells in culture. Ea.hy926 endothelial cells were plated onto Matrigel and after 2 days were treated with VEGF at 10 ng/ml (~ 0.5 nM), Pam3CSK4 at 7 μ M, or recombinant proteins at 1 μ g/ml (~ 20 nM). Additional wells were infected with *B. burgdorferi* at an MOI of 10. Cellular growth and structure formation were quantified visually over 7 to 10 days, depending on the lot of Matrigel; three random fields per sample were assessed at each time point. In some experiments, both purified protein and bacterial strain sets were included; in some experiments only bacterial sets or recombinant protein sets were compared. In all experiments the positive and negative controls (VEGF and no treatment, respectively) were included. (A) Representative fields 3 days after the addition of bacteria or VEGF. (B) Because the data sets (photographs and cell counts) are too large to present for all comparisons, the *P* values (by one-way repeated-measures ANOVA followed by Dunnett's posttest) are shown for the comparisons tested in a single large representative experiment. VEGF and MBP-OspC-treated cells, and cells infected with the WT or Δ C/pOspC *B. burgdorferi* strain, were significantly different from the untreated controls; cells treated with MBP- β gal (a control for MBP-OspC) or infected with the Δ C *B. burgdorferi* strain were not. Differences were amplified with increasing incubation times until the cell density reached a point at which cell numbers could not be reliably quantified. Differences between the effects of *B. burgdorferi* and the cell growth rate and degree of structure formation depended to some extent on the lot of Matrigel. ND, not determined; NA, not applicable. An asterisk denotes a condition under which statistically significant differences appeared to vary with the lot of Matrigel.

cantly less effective than the positive control, VEGF, and than the WT bacteria. However, this result appears to vary with the lot of Matrigel used, which affects the growth rate of the cells overall. Consistent with this intermediate activity, purified MBP-OspC, but not the control MBP- β gal, induced endothelial growth and structure formation. Together, these results suggest that OspC participates in this activity but that other *B. burgdorferi* factors also contribute. The lipopeptide Pam3CSK4 also showed an intermediate activation of endothelial cell growth and tubule formation but at a higher molarity. VEGF was not detected by ELISA in the supernatants from any wells (with or without Matrigel) infected with *B. burgdorferi* (data not shown), suggesting that any VEGF that is produced might rapidly be taken up by the cells or degraded or that the bacteria themselves mimic VEGF in this system. Laboratory *Escherichia coli* K-12 did not promote endothelial cell growth and in fact caused the death of the cells within 3 days (data not shown).

DISCUSSION

OspC is an outer surface protein that is abundantly expressed by *Borrelia burgdorferi* as the organism enters the mammalian host. OspC-deficient bacteria cannot establish infection even in the immunodeficient mouse strains that have been tested (25, 57). It has also been shown that OspC expression is required only for a short time in order for the bacteria to establish infection when introduced from either *in vitro* culture or ticks (59, 61). The exact function of the protein that makes it so important for the bacteria is not known, but the available data suggested that OspC might interact with an as-yet-unidentified component of the mammalian host response at the initial stages of infection.

The data presented here are consistent with previously published evidence (25, 54, 57) that the presence of OspC does not alter the host innate immune response to the bacteria. Macrophages *in vitro* have the same survival rates when infected

with WT and ΔC bacteria and secrete the same cytokines at similar levels. These data suggest that OspC does not play a role in the interaction of the bacteria with the macrophages, even though the latter are one of the first types of cells to respond to the site of infection (42). It may be argued other cell types, including those of the innate immune system, may be affected by the expression of OspC, in particular with regard to the production of MCP-1, as bone marrow-derived macrophages in culture did not respond to *B. burgdorferi* in terms of MCP-1 production. Neutrophils are also found at the site of infection (64), and in a recent paper Sarkar and colleagues (54) investigated the possible *in vitro* effects on *B. burgdorferi* of the antimicrobial peptide cathelicidin, which is produced by neutrophils and keratinocytes and so is inducible in the skin in response to infection (6). However, there were no differences between the WT and ΔC *B. burgdorferi* strains in their resistances to cathelicidin (54).

Consistent with the *in vitro* results, the presence or absence of OspC did not significantly affect either the recruitment of macrophages and neutrophils or the levels of the chemokines KC and MCP-1 within the first 2 days of infection by *B. burgdorferi*. Statistically significantly higher numbers of monocytes, and of MCP-1 and KC, were observed at day 7 postinfection, when the ΔC bacteria had been cleared. Thus, the presence of monocytes, MCP-1, and KC relates to the presence of the bacteria and not directly to the expression of OspC by the bacteria. MCP-1 mRNA has been detected in erythema migrans lesions of human patient skin (67) and was shown to be induced in infections *in vitro* (10), but to our knowledge this is the first report that MCP-1 is produced in the skin of mice at the site of *B. burgdorferi* infection. However, the way in which the host initially responds does not significantly depend on the presence or absence of OspC production by *B. burgdorferi*. The same cytokines are present at the same levels, and the histopathology results support these data.

From the data that we and others have collected, it appears that host immunological restriction does not account for the inability of the ΔC bacteria to survive *in vivo*. Instead, OspC could play roles in other interactions with the host to facilitate the survival and dissemination of *B. burgdorferi* or in bacterial adaptation to their new environment in the mammalian host. OspC expression is essential during the first few days, but its constant expression is detrimental for the bacteria (63). Although this remains speculative at this point, one possible role for OspC might be in sensing the change in the environment as the bacteria move from the tick to the skin of the mammalian host. This possibility, however, is not consistent with previous results in which the ΔC mutant was able to survive in a protected environment in the peritoneal cavity (57). It is possible, however, that any adaptation mediated through OspC may not be discernible in such an environment.

Consistent with this possibility, an alternative possible role for OspC is in interactions with specific host tissue components. The substitution for OspC by other lipoproteins allowed the bacteria to survive in the skin but did not complement the establishment of infection at distant sites such as the heart, and the dissemination rate was reduced (62). A direct interaction of the bacteria with a mammalian component might be necessary for the bacteria to sense the new environment and to colonize specific tissue sites in which that component may be

particularly abundant. This possibility is supported by our previous observation that OspC-bearing phage clones were efficiently selected for binding to the vasculature in living mice, particularly in the heart (2). In addition, both groups that determined the structure predicted the presence of a binding site for an unknown ligand (21, 34). In fact, one particular residue selected for site-directed mutagenesis on the basis of location within a putative ligand-binding domain was demonstrated to be essential for *B. burgdorferi* infectivity (17). The molecule to which OspC binds in the mammalian host might be a signal to which the bacteria respond by changing the composition of the outer membrane, either at the transcriptional or at the posttranscriptional level. For example, the induction of antigenic variation in VlsE is critical for *B. burgdorferi* survival in the mammalian host (16, 37) and relies on an as-yet-unknown signal in the mammalian environment. In fact, the interaction of *B. burgdorferi* with endothelial cells *in vitro* was reported previously to induce *vlsE* expression (31), although VlsE expression is not equivalent to variation.

A role for OspC in interactions of *B. burgdorferi* with the vasculature is supported by two independent lines of evidence. OspC was selected as a candidate adhesin using *in vivo* phage display (2), and data presented here suggest that OspC may affect host barriers to disseminated infection by contributing to the induction of VEGF at the site of infection. The coinjection of VEGF with the ΔC *B. burgdorferi* strain did not, however, "rescue" the virulence defect of the ΔC *B. burgdorferi* strain in C3H/HeN mice (data not shown). One interpretation of these data is that VEGF is a marker for *B. burgdorferi*-host interactions but is not the critical factor in mediating the establishment of infection by *B. burgdorferi*. Interactions with the vasculature are a mammal-specific signal and can determine tissue tropism, as the endothelia of different sites are different (50–52). The data presented here, together with previously reported results enumerated above, suggest that *B. burgdorferi* may manipulate the host vasculature to its own advantage and that OspC may be an important factor in this manipulation. Based on the results presented in Fig. 4 and 5, however, OspC is not the sole contributor to this activity, and additional *B. burgdorferi* factors await identification. The use of state-of-the-art approaches such as intravital microscopy (45, 48) should provide further illumination of the role of OspC in mediating *B. burgdorferi* interactions with the mammalian host.

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REFERENCES

- Alverson, J., S. F. Bundle, C. D. Sohaskey, M. C. Lybecker, and D. S. Samuels. 2003. Transcriptional regulation of the *ospAB* and *ospC* promoters from *Borrelia burgdorferi*. *Mol. Microbiol.* 48:1665–1677.
- Antonara, S., R. M. Chafel, M. Lafrance, and J. Coburn. 2007. *Borrelia burgdorferi* adhesins identified using *in vivo* phage display. *Mol. Microbiol.* 66:262–276.
- Behera, A. K., E. Hildebrand, S. Uematsu, S. Akira, J. Coburn, and L. T. Hu.

2006. Identification of a TLR-independent pathway for *Borrelia burgdorferi*-induced expression of matrix metalloproteinases and inflammatory mediators through binding to integrin $\alpha 3 \beta 1$. *J. Immunology* **177**:657–664.
4. Benhnia, M. R., D. Wroblewski, M. N. Akhtar, R. A. Patel, W. Lavezzi, S. C. Gangloff, S. M. Goyert, M. J. Caimano, J. D. Radolf, and T. J. Sellati. 2005. Signaling through CD14 attenuates the inflammatory response to *Borrelia burgdorferi*, the agent of Lyme disease. *J. Immunol.* **174**:1539–1548.
5. Berger, B. W., M. H. Kaplan, I. R. Rothenberg, and A. G. Barbour. 1985. Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. *J. Am. Acad. Dermatol.* **13**: 444–449.
6. Braff, M. H., M. Zaiou, J. Fierer, V. Nizet, and R. L. Gallo. 2005. Keratinocyte production of cathelicidin provides direct activity against bacterial skin pathogens. *Infect. Immun.* **73**:6771–6781.
7. Brown, C. R., V. A. Blahou, and C. M. Loiacono. 2003. Susceptibility to experimental Lyme arthritis correlates with KC and monocyte chemoattractant protein-1 production in joints and requires neutrophil recruitment via CXCR2. *J. Immunol.* **171**:893–901.
8. Brown, J. P., J. F. Zachary, C. Teuscher, J. J. Weis, and R. M. Wooten. 1999. Dual role of interleukin-10 in murine Lyme disease: regulation of arthritis severity and host defense. *Infect. Immun.* **67**:5142–5150.
9. Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. Lyme disease—a tick-borne spirochetosis? *Science* **216**: 1317–1319.
10. Burns, M. J., T. J. Sellati, E. I. Teng, and M. B. Furie. 1997. Production of interleukin-8 (IL-8) by cultured endothelial cells in response to *Borrelia burgdorferi* occurs independently of secretion of IL-1 and tumor necrosis factor and is required for subsequent transendothelial migration of neutrophils. *Infect. Immun.* **65**:1217–1222.
11. Caimano, M. J., C. H. Eggers, K. R. Hazlett, and J. D. Radolf. 2004. RpoS is not central to the general stress response in *Borrelia burgdorferi* but does control expression of one or more essential virulence determinants. *Infect. Immun.* **72**:6433–6445.
12. Caimano, M. J., R. Iyer, C. H. Eggers, C. Gonzalez, E. A. Morton, M. A. Gilbert, I. Schwartz, and J. D. Radolf. 2007. Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle. *Mol. Microbiol.* **65**:1193–1217.
13. CDC. 2009. Lyme disease incidence by state, 2004–2008. CDC, Atlanta, GA. http://www.cdc.gov/ncidod/dvbid/lyme/ld_IncidenceRatesbyState20042008.htm.
14. CDC. 2009. Lyme disease. CDC, Atlanta, GA. <http://wwwnc.cdc.gov/travel/yellowbook/2010/chapter-5/lyme-disease.aspx>.
15. Craig-Mylus, K., G. F. Weber, J. E. Coburn, and L. Glickstein. 2005. *Borrelia burgdorferi*, an extracellular pathogen, circumvents osteopontin in inducing an inflammatory cytokine response. *J. Leukoc. Biol.* **77**:710–718.
16. Dresser, A. R., P. O. Hardy, and G. Chaconas. 2009. Investigation of the genes involved in antigenic switching at the *vlsE* locus in *Borrelia burgdorferi*: an essential role for the RuvAB branch migrase. *PLoS Pathog.* **5**:e1000680.
17. Earnhart, C. G., D. V. Leblanc, K. E. Alix, D. C. Desrosiers, J. D. Radolf, and R. T. Marconi. 2010. Identification of residues within ligand-binding domain 1 (LBD1) of the *Borrelia burgdorferi* OspC protein required for function in the mammalian environment. *Mol. Microbiol.* **76**:393–408.
18. Edgell, C. J., J. E. Haizlip, C. R. Bagnall, J. P. Packenham, P. Harrison, B. Wilbourn, and V. J. Madden. 1990. Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. *In Vitro Cell. Dev. Biol.* **26**:1167–1172.
19. Edgell, C. J., C. C. McDonald, and J. B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. U. S. A.* **80**:3734–3737.
20. Eggers, C. H., M. J. Caimano, and J. D. Radolf. 2004. Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. *J. Bacteriol.* **186**:7390–7402.
21. Eicken, C., V. Sharma, T. Klabunde, R. T. Owens, D. S. Pikas, M. Hook, and J. C. Sacchettini. 2001. Crystal structure of Lyme disease antigen outer surface protein C from *Borrelia burgdorferi*. *J. Biol. Chem.* **276**:10010–10015.
22. Elias, A. F., P. E. Stewart, D. Grimm, M. J. Caimano, C. H. Eggers, K. Tilly, J. L. Bono, D. R. Akins, J. D. Radolf, T. G. Schwan, and P. A. Rosa. 2002. Clonal polymorphism of *Borrelia burgdorferi* strain B31 MF: implications for mutagenesis in an infectious strain background. *Infect. Immun.* **70**:2139–2150.
23. Esteve-Gassent, M. D., N. L. Elliott, and J. Seshu. 2009. *sodA* is essential for virulence of *Borrelia burgdorferi* in the murine model of Lyme disease. *Mol. Microbiol.* **71**:594–612.
24. Glickstein, L. J., and J. L. Coburn. 2006. Association of macrophage inflammatory response and cell death after *in vitro* *Borrelia burgdorferi* infection with arthritis resistance. *Am. J. Trop. Med. Hyg.* **75**:964–967.
25. Grimm, D., K. Tilly, R. Byram, P. E. Stewart, J. G. Krum, D. M. Bueschel, T. G. Schwan, P. F. Policastro, A. F. Elias, and P. A. Rosa. 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. *Proc. Natl. Acad. Sci. U. S. A.* **101**:3142–3147.
26. Guerau-de-Arellano, M., and B. T. Huber. 2005. Chemokines and Toll-like receptors in Lyme disease pathogenesis. *Trends Mol. Med.* **11**:114–120.
27. Habicht, G. S., L. I. Katona, and J. L. Benach. 1991. Cytokines and the pathogenesis of neuroborreliosis: *Borrelia burgdorferi* induces glioma cells to secrete interleukin-6. *J. Infect. Dis.* **164**:568–574.
28. Hodzic, E., S. Feng, K. J. Freet, and S. W. Barthold. 2003. *Borrelia burgdorferi* population dynamics and prototype gene expression during infection of immunocompetent and immunodeficient mice. *Infect. Immun.* **71**:5042–5055.
29. Hovius, J. W., M. F. Bijlsma, G. J. van der Windt, W. J. Wiersinga, B. J. Boukens, J. Coumou, A. Oei, R. de Beer, A. F. de Vos, C. van't Veer, A. P. van Dam, P. Wang, E. Fikrig, M. M. Levi, J. J. Roelofs, and T. van der Poll. 2009. The urokinase receptor (uPAR) facilitates clearance of *Borrelia burgdorferi*. *PLoS Pathog.* **5**:e1000447.
30. Hubner, A., X. Yang, D. M. Nolen, T. G. Popova, F. C. Cabello, and M. V. Norgard. 2001. Expression of *Borrelia burgdorferi* OspC and DbpA is controlled by a RpoN-RpoS regulatory pathway. *Proc. Natl. Acad. Sci. U. S. A.* **98**:12724–12729.
31. Hudson, C. R., J. G. Frye, F. D. Quinn, and F. C. Gherardini. 2001. Increased expression of *Borrelia burgdorferi* *vlsE* in response to human endothelial cell membranes. *Mol. Microbiol.* **41**:229–239.
32. Jewett, M. W., K. Lawrence, A. C. Bestor, K. Tilly, D. Grimm, P. Shaw, M. VanRaden, F. Gherardini, and P. A. Rosa. 2007. The critical role of the linear plasmid lp36 in the infectious cycle of *Borrelia burgdorferi*. *Mol. Microbiol.* **64**:1358–1374.
33. Kubota, Y., H. K. Kleinman, G. R. Martin, and T. J. Lawley. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107**:1589–1598.
34. Kumar, D., S. Eswaramoorthy, B. J. Luft, S. Koide, J. J. Dunn, C. L. Lawson, and S. Swaminathan. 2001. Crystal structure of outer surface protein C (OspC) from the Lyme disease spirochete, *Borrelia burgdorferi*. *EMBO J.* **20**:971–978.
35. Lazarus, J. J., M. A. Kay, A. L. McCarter, and R. M. Wooten. 2008. Viable *Borrelia burgdorferi* enhances interleukin-10 production and suppresses activation of murine macrophages. *Infect. Immun.* **76**:1153–1162.
36. Liang, F. T., M. B. Jacobs, L. C. Bowers, and M. T. Philipp. 2002. An immune evasion mechanism for spirochetal persistence in Lyme borreliosis. *J. Exp. Med.* **195**:415–422.
37. Lin, T., L. Gao, D. G. Edmondson, M. B. Jacobs, M. T. Philipp, and S. J. Norris. 2009. Central role of the Holliday junction helicase RuvAB in *vlsE* recombination and infectivity of *Borrelia burgdorferi*. *PLoS Pathog.* **5**:e1000679.
38. Lybecker, M. C., and D. S. Samuels. 2007. Temperature-induced regulation of RpoS by a small RNA in *Borrelia burgdorferi*. *Mol. Microbiol.* **64**:1075–1089.
39. Ma, Y., K. P. Seiler, K. F. Tai, L. Yang, M. Woods, and J. J. Weis. 1994. Outer surface lipoproteins of *Borrelia burgdorferi* stimulate nitric oxide production by the cytokine-inducible pathway. *Infect. Immun.* **62**:3663–3671.
40. Maeshima, Y., M. Manfredi, C. Reimer, K. A. Holthaus, H. Hoyer, B. R. Chandamuri, S. Kharbanda, and R. Kalluri. 2001. Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. *J. Biol. Chem.* **276**:15240–15248.
41. Matalaka, K. Z., M. F. Tutunji, M. Abu-Baker, and Y. Abu Baker. 2005. Measurement of protein cytokines in tissue extracts by enzyme-linked immunosorbent assays: application to lipopolysaccharide-induced differential milieu of cytokines. *Neuro Endocrinol. Lett.* **26**:231–236.
42. Montgomery, R. R., D. Lusitani, A. de Boisfleury Chevance, and S. E. Malawista. 2002. Human phagocytic cells in the early innate immune response to *Borrelia burgdorferi*. *J. Infect. Dis.* **185**:1773–1779.
43. Montgomery, R. R., S. E. Malawista, K. J. Feen, and L. K. Bockenstedt. 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* *ex vivo*: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. *J. Exp. Med.* **183**:261–269.
44. Montgomery, R. R., M. H. Nathanson, and S. E. Malawista. 1994. Fc- and non-Fc-mediated phagocytosis of *Borrelia burgdorferi* by macrophages. *J. Infect. Dis.* **170**:890–893.
45. Moriarty, T. J., M. U. Norman, P. Colarusso, T. Bankhead, P. Kubes, and G. Chaconas. 2008. Real-time high resolution 3D imaging of the Lyme disease spirochete adhering to and escaping from the vasculature of a living host. *PLoS Pathog.* **4**:e1000090.
46. Murthy, P. K., V. A. Dennis, B. L. Lasater, and M. T. Philipp. 2000. Interleukin-10 modulates proinflammatory cytokines in the human monocytic cell line THP-1 stimulated with *Borrelia burgdorferi* lipoproteins. *Infect. Immun.* **68**:6663–6669.
47. Myers, T. A., D. Kaushal, and M. T. Philipp. 2009. Microglia are mediators of *Borrelia burgdorferi*-induced apoptosis in SH-SY5Y neuronal cells. *PLoS Pathog.* **5**:e1000659.
48. Norman, M. U., T. J. Moriarty, A. R. Dresser, B. Millen, P. Kubes, and G. Chaconas. 2008. Molecular mechanisms involved in vascular interactions of the Lyme disease pathogen in a living host. *PLoS Pathog.* **4**:e1000169.
49. Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig. 2004. OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. *J. Clin. Invest.* **113**:220–230.

50. Pasqualini, R., and W. Arap. 2002. Profiling the molecular diversity of blood vessels. Cold Spring Harbor Symp. Quant. Biol. **67**:223–225.
51. Pasqualini, R., and W. Arap. 2002. Translation of vascular diversity into targeted therapeutics. Ann. Hematol. **81**(Suppl. 2):S66–S67.
52. Pasqualini, R., W. Arap, and D. M. McDonald. 2002. Probing the structural and molecular diversity of tumor vasculature. Trends Mol. Med. **8**:563–571.
53. Potter, M. R., S. R. Rittling, D. T. Denhardt, R. J. Roper, J. H. Weis, C. Teuscher, and J. J. Weis. 2002. Role of osteopontin in murine Lyme arthritis and host defense against *Borrelia burgdorferi*. Infect. Immun. **70**:1372–1381.
54. Sarkar, A., K. Tilly, P. Stewart, A. Bestor, J. M. Battisti, and P. A. Rosa. 2009. *Borrelia burgdorferi* resistance to a major skin antimicrobial peptide is independent of outer surface lipoprotein content. Antimicrob. Agents Chemother. **53**:4490–4494.
55. Schwan, T. G., and J. Piesman. 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. **38**:382–388.
56. Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa. 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. U. S. A. **92**:2909–2913.
57. Stewart, P. E., X. Wang, D. M. Bueschel, D. R. Clifton, D. Grimm, K. Tilly, J. A. Carroll, J. J. Weis, and P. A. Rosa. 2006. Delineating the requirement for the *Borrelia burgdorferi* virulence factor OspC in the mammalian host. Infect. Immun. **74**:3547–3553.
58. Swanson, M. S., and R. R. Isberg. 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. Infect. Immun. **63**:3609–3620.
59. Tilly, K., A. Bestor, D. P. Dulebohn, and P. A. Rosa. 2009. OspC-independent infection and dissemination by host-adapted *Borrelia burgdorferi*. Infect. Immun. **77**:2672–2682.
60. Tilly, K., A. Bestor, M. W. Jewett, and P. Rosa. 2007. Rapid clearance of Lyme disease spirochetes lacking OspC from skin. Infect. Immun. **75**:1517–1519.
61. Tilly, K., J. G. Krum, A. Bestor, M. W. Jewett, D. Grimm, D. Bueschel, R. Byram, D. Dorward, M. J. Vanraden, P. Stewart, and P. Rosa. 2006. *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. Infect. Immun. **74**:3554–3564.
62. Xu, Q., K. McShan, and F. T. Liang. 2008. Essential protective role attributed to the surface lipoproteins of *Borrelia burgdorferi* against innate defenses. Mol. Microbiol. **69**:15–29.
63. Xu, Q., S. V. Seemanapalli, K. McShan, and F. T. Liang. 2006. Constitutive expression of outer surface protein C diminishes the ability of *Borrelia burgdorferi* to evade specific humoral immunity. Infect. Immun. **74**:5177–5184.
64. Xu, Q., S. V. Seemanapalli, K. E. Reif, C. R. Brown, and F. T. Liang. 2007. Increasing the recruitment of neutrophils to the site of infection dramatically attenuates *Borrelia burgdorferi* infectivity. J. Immunol. **178**:5109–5115.
65. Yang, X. F., S. M. Alani, and M. V. Norgard. 2003. The response regulator Rrp2 is essential for the expression of major membrane lipoproteins in *Borrelia burgdorferi*. Proc. Natl. Acad. Sci. U. S. A. **100**:11001–11006.
66. Yang, X. F., M. C. Lybecker, U. Pal, S. M. Alani, J. Blevins, A. T. Revel, D. S. Samuels, and M. V. Norgard. 2005. Analysis of the ospC regulatory element controlled by the RpoN-RpoS regulatory pathway in *Borrelia burgdorferi*. J. Bacteriol. **187**:4822–4829.
67. Zhao, Z., B. McCloud, R. Fleming, and M. S. Klempner. 2007. *Borrelia burgdorferi*-induced monocyte chemoattractant protein-1 production *in vivo* and *in vitro*. Biochem. Biophys. Res. Commun. **358**:528–533.

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