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Presence of Arp Specifically Contributes to Joint Tissue Edema Associated with Early-Onset Lyme Arthritis

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Antiserum to the *Borrelia burgdorferi* arthritis-related protein, Arp, has been shown to prevent or reduce arthritis in immunodeficient mice. To directly investigate the requirement for this lipoprotein in the generation of Lyme arthritis, we utilized targeted deletion to generate a *B. burgdorferi* clone that lacked only the *arp* gene locus. Infection of Lyme disease-susceptible C3H/HeN mice with the *arp* deletion mutant demonstrated significantly reduced tibiotarsal joint swelling during the first 6 weeks of infection compared to a wild-type control. The severity of joint swelling was restored to wild-type levels in mice infected with an *arp* mutant clone complemented in *cis*. Interestingly, the reduced swelling of joint tissues exhibited by mice infected with the *arp* deletion mutant did not directly correspond to reduced underlying arthritis. Histopathology data at 2 weeks postinfection showed some reduction in arthritis severity caused by the *arp* mutant clone; however, by 8 weeks, no significant difference was observed between joint tissues infected by the wild-type or *arp* mutant clones. The spirochete load in the joint tissues of mice infected with the *arp* mutant was found to be greater than that exhibited by the wild-type control. Our findings demonstrate that this lipoprotein contributes to the generation of early-onset joint swelling and suggests that *arp* expression has a negative secondary effect on total spirochete numbers in joint tissues.

Lyme disease is caused by infection with the tick-transmitted spirochete *Borrelia burgdorferi*. It is currently the most common vector-borne disease in the Northern Hemisphere, occurring in parts of North America, Europe, and Asia (1, 2). The disease affects a wide range of mammals, including humans, horses, and dogs (3–7), and ticks of the genus *Ixodes* are the primary vectors of the disease (8). A blood meal by an infected tick is followed by a strong immune response, and infection results in a multisystem disease characterized by damage to the central nervous system and various organs, including the heart, eyes, and joints. Despite a robust humoral and cellular response, chronic and persistent infection can often result. Among the afflicted tissue sites, the joints are a major site of inflammation (9), and subacute arthritis occurs in 60% of untreated individuals (10, 11). In humans, this subacute arthritis can often develop into a chronic form characterized by bacterial persistence that is often unresponsive to antibiotics. Important in disease pathology is the large number of plasmid-encoded surface lipoproteins that have the potential to trigger host immune responses (12–14). Previous studies have demonstrated that lipoproteins or their derivatives activate endothelial cells, neutrophils, macrophages, and B lymphocytes *in vitro* and can introduce localized inflammatory infiltrate into joints and dermal sites *in vivo* (15–20).

A number of genes coding for *B. burgdorferi* lipoproteins have been shown to be preferentially upregulated at various times in different tissue sites during infection of the mammalian host (21–24). One such gene, coding for the *a*rthritis-*r*elated *p*rotein (Arp), resides on linear plasmid 28-1 (lp28-1) (25) and has been shown to be upregulated in the joints of infected immunocompetent mice (21). Previous studies demonstrated that *B. burgdorferi*-infected severe combined immunodeficient (SCID) mice treated with Arp antiserum exhibit reduced arthritis severity without affecting the status of infection, suggesting Arp as a target for immune-mediated resolution of Lyme arthritis (25). In a recent study, published during the preparation of the manuscript, it was shown that deletion of *arp* by allelic exchange resulted in reduced arthritis severity and spirochete load in immunocompetent C3H mice (26). In the present study, we generated an *arp* mutant through telomere-targeted deletion and infected immunocompetent C3H/HeN mice to determine if the absence of Arp had an effect on both joint swelling and immune cell infiltration into the joint tissues of mice. The results show that deletion of *arp* led to a significant reduction in measurable tibiotarsal (ankle) joint swelling during the early onset of infection by the mutant clone. Interestingly, this reduction in swelling did not correspond to a decrease in overall immune cell infiltration and subsequent joint pathology. Additionally, the spirochete load in infected joint tissues was shown to be higher in mice infected with the mutant clone than in mice infected with the wild type (WT).

MATERIALS AND METHODS

***B. burgdorferi* strains and culture conditions.** *Borrelia burgdorferi* B31-5A4 (wild type) was a kind gift from Steve Norris. The clones described in the study were generated from the above-mentioned B31 strain, whose infectivity and plasmid profile had already been determined (Table 1) (27). All *B. burgdorferi* clones were cultivated in liquid Barbour-Stoenner-Kelly II (BSK-II) medium supplemented with 6% rabbit serum (Cedarlane Laboratories, Burlington, NC) and incubated at 35°C in 2.5% CO_2. The mutant strains were grown with kanamycin (200 μg/ml) or gentamicin (100 μg/ml), as indicated. Cell densities and growth phases were monitored by dark-field microscopy and enumerated using a Petroff-Hausser counting chamber.

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TABLE 1 Strains used in this study

B. burgdorferi B31 clone	*arp* presence[a]	Reference or source
5A4 WT	+	27
5A4Δ*arp* (ΔArp)	−	This study
5A4Δ*arp*::*arp* (cArp)	+	This study

[a] +, present; −, absent.

Generation of *arp* deletion and complement mutant clones. For the targeted deletion of *arp*, a 1,059-bp region upstream of the *arp* gene locus (coordinates 1471 to 2484 of the annotated lp28-1 sequence; NCBI reference sequence NC_001851.2 [http://www.ncbi.nlm.nih.gov/]) was PCR amplified using primers P270 and P271 (Table 2). The resulting DNA product was then cloned into the pGCL47-4 plasmid, which carries a *flgB*p-driven kanamycin gene and a 70-bp replicated telomere (*rtel*) from the left end of linear plasmid 17 (lp17) (28) in order to generate pPH12. The resulting plasmid was then transformed into recombination-deficient *Escherichia coli* Ec19 competent cells (*recA recB21 recC22 sbcB15 hsdR* F^- *proA2 his4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 str-31 tsx-33*), which are derivatives of *E. coli* DB1256 (29). Plasmid DNA isolated from individual *E. coli* clones was verified for correct size and orientation by restriction digestion, and a functional *rtel* was assessed using a ResT assay, as previously described (30), before transformation into *B. burgdorferi* cells.

The in *cis arp* complement *B. burgdorferi* clone was generated by amplifying *arp*, including 400 bp of upstream sequence (containing the native promoter element), followed by insertion into pPH12 at the NheI restriction site to create the complementation construct pPH25. The kanamycin resistance gene in this construct was replaced by cloning in the *aacC1* gene conferring gentamicin resistance at the NgoMIV and NheI restriction sites. The resulting plasmid DNA construct was then transformed into *E. coli* and cultured under gentamicin selection. The plasmids isolated from verified *E. coli* clones were then used to transform the *arp* mutant clone.

***B. burgdorferi* transformation.** *B. burgdorferi* B31-5A4 wild-type or *arp* deletion mutant cells were electroporated and cultured as previously described (28). DNA from culture-positive wells was extracted using a DNeasy Blood and Tissue kit (Qiagen, Germantown, MD) and used for PCR analysis to confirm the presence of the antibiotic resistance gene and the presence or absence of *arp* (Table 2 lists the primers). The plasmid content for each verified transformant was determined by PCR using plasmid-specific primers, as previously described (27).

Southern blot analysis. Total plasmid DNA was extracted from *B. burgdorferi* clones using the Plasmid Midi kit (Qiagen) and separated on a 1% agarose gel at 80 V for 23 h (250 ng of DNA was used per lane). The DNA was then transferred onto a nylon membrane and hybridized with digoxigenin (DIG)-labeled probes following the manufacturer's guidelines (Roche, Indianapolis, IN).

Murine infection. All animal infections were carried out in accordance with approved protocols from the Institutional Animal Care and Use Committee (IACUC) of Washington State University. Male C3H/HeN mice (Harlan, Indianapolis, IN) at 7 weeks of age were infected by subcutaneous needle inoculation with 10^5 total spirochetes. Five mice per experimental group were treated at each time point. *B. burgdorferi* clones were passaged no more than two times *in vitro* from frozen glycerol stock prior to use in mouse infection studies. Infection was monitored by culturing either blood samples or ear biopsy specimens at the indicated times postinfection. Blood and tissue samples were cultured in BSK-II medium containing *Borrelia* antibiotic cocktail (0.02 mg/ml phosphomycin, 0.05 mg/ml rifampin, and 2.5 μg/ml amphotericin B). Disease progression was noted weekly by visual examination and digital-caliper measurements. At 2, 4, and 8 weeks postinfection, mice were sacrificed, and ear, heart, bladder, and joint tissues were obtained aseptically and cultured in BSK-II medium containing *Borrelia* antibiotic cocktail. Dark-field microscopy was used to determine the presence or absence of viable spirochetes for each cultured tissue sample.

Measurement of tibiotarsal joints. Mice were anesthetized with isoflurane prior to joint measurements taken with a digital metric caliper (Mitutoyo, Tokyo, Japan). Measurements were taken in the anterior-to-posterior position with the knee extended, through the thickest portion of the ankle (31). The ankle diameter increase was obtained by subtracting the preinfection joint diameter from the measurements taken at each week for 4 or 8 weeks.

Histology of tibiotarsal joints. At 2, 4, and 8 weeks postinfection, 5 mice from each experimental group were sacrificed, and the tibiotarsal joint displaying the greatest swelling was collected for histopathology. The joints were immediately fixed in 10% neutral buffered formalin. Sections from the decalcified and paraffin-embedded joints were then stained with hematoxylin and eosin. Sections were scored blindly from 0 to 4, and the scores were defined as follows: 0, no change (no inflammation); 1, minimal change (less than 1% of the area of the tibiotarsal joint infiltrated with leukocytes); 2, mild change (1 to 25% of the area infiltrated with leukocytes); 3, moderate change (25 to 50% of the area infiltrated with leukocytes, often with synovial hyperplasia, periarticular fibrosis, and/or min-

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Description
P54	CATATGAGCCATATTCAACGGGAAACG	Forward primer for *kan* screening and probe generation
P55	AAAGCCGTTTCTGTAATGAAGGAG	Reverse primer for *kan* screening and probe generation
P91	CGCAGCAGCAACGATGTTAC	Forward primer for *gent* screening
P92	CTTGCACGTAGATCACATAAGC	Reverse primer for *gent* screening
P202	AGAGGGAAATCGTGCGTGAC	Forward primer for qPCR of mouse *actB*
P203	CAATAGTGATGACCTGGCCGT	Reverse primer for qPCR of mouse *actB*
P204	CACTGCCGCATCCTCTTCCTCCC	Probe primer for qPCR of mouse *actB*
P270	CCGGGTACCCAATCGGATTTTTAACTTAAAGTCG	Forward primer for *arp* targeted deletion with KpnI site
P271	CCGGAGCTCGACAATCTTGTTACTAAGATTGATAACG	Reverse primer for *arp* targeted deletion with SacI site
P302	CATGCTCCAAACTCAAAAATTG	Forward primer for *arp* screening and probe generation
P303	GGGTGTGTAATTTTTTCTTCAACTTC	Reverse primer for *arp* screening and probe generation
P357	CCGGCTAGCGATGTAGAAAATGATGTAGCCTCTACTAAATAATGTG	Reverse primer for *arp* complement generation with NheI site
P360	CCGGCTAGCTGCAAAAATTTGTATAATCTAAAATTATACATTAATG	Forward primer for *arp* complement generation with NheI site
P359	CCGGGATCCTTAACTTAAACCCTTTACACTTTCTTCG	Reverse primer *arp* recombinant protein with BamHI site
P361	CCGCATATGAAATTTGATAGTCTTAATTTATCTACAAAAAGC	Forward primer *arp* recombinant protein with NdeI site
P411	GAGTTTCTGGTAAGATTAATGCTC	Forward primer for qPCR of *flaB*
P412	CATTTAAATTCCCTTCTGTTGTCTGA	Reverse primer for qPCR of *flaB*
P413	AGAGGTTTGTCACAAGCTTCTAGAAATACTTCAAAGGC	Probe for qPCR of *flaB*

imal to mild exudate within the joint and/or tendon sheath); 4, severe change (more than 50% of the area infiltrated as described above plus moderate to severe exudate within the joint and/or tendon sheath, often also with superficial inflammation of the bone). The average score for each group at each time point was obtained, and significant differences were determined.

qPCR analysis. Mice infected with experimental strains for quantitative-PCR (qPCR) analysis were sacrificed at either 2, 4, or 8 weeks, and the most swollen tibiotarsal joint was collected from each mouse. Four mice per group were used at the 2-week time point and 5 mice per group at the 4- and 8-week time points. The samples were immediately snap-frozen in liquid nitrogen and ground thoroughly with a mortar and pestle. DNA was extracted by using the DNeasy Minikit following the manufacturer's instructions (Qiagen) and stored at −20°C. Plasmid constructs containing the mouse β-actin gene (*actB*) and the *B. burgdorferi flaB* gene were generated to create absolute standards. The sequences of primers and internal probes were described previously for *flaB* (21) and *actB* (32) (Table 2). Primers for *flaB* and *actB* amplified a 115-bp and a 138-bp DNA fragment, respectively. Each of these DNA fragments was separately cloned into pJET2.1 (Fermentas) and verified by DNA sequencing. DNA concentrations were determined by measuring the optical density at 260-nm wavelength and converted to the respective copy numbers.

qPCR analyses were performed using the CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). qPCR was carried out in 20-μl reaction mixtures containing 1× SsoFast Probes Supermix (Bio-Rad). DNA standards containing 10^4 to 10^0 copies/well of the *B. burgdorferi flaB* gene or mouse *actB* containing 10^7 to 10^3 copies/well were run on each plate. Both standards and samples were amplified in triplicate. The amplification program was performed as follows: (i) heating at 95°C for 2 min for polymerase activation and DNA denaturation and (ii) amplification for 40 cycles with denaturation at 95°C for 10 s and extension and annealing at 60°C. Plate reading was done at 60°C. The mean DNA copy numbers of *flaB* and *actB* for each DNA sample were calculated from triplicate wells. Tissue spirochete levels were converted to *flaB* DNA copy numbers per 10^6 *actB* DNA copies. The average copy number for each group at each time point was obtained, and significant differences were determined.

Statistical analysis. SigmaPlot 11.0 software was used for all statistical data analysis. The Student *t* test was used for the comparison of two groups, while one-way analysis of variance (ANOVA) was used when comparing data sets comprising more than two groups. This was followed by the all-pairwise multiple-comparison procedure (Holm-Sidak method) if there were significant differences among treatment means ($P < 0.05$). The Kruskal-Wallis ANOVA on ranks was applied in cases where the normality test failed, followed by the all-pairwise multiple-comparison procedure (Tukey test; $P < 0.05$).

RESULTS

Generation of *B. burgdorferi arp* deletion and complement clones. Antiserum to Arp has been shown to prevent or reduce arthritis in immunodeficient mice (25), suggesting involvement of the lipoprotein in the generation of Lyme arthritis. To investigate the requirement for Arp in joint inflammation associated with *B. burgdorferi* infection, a mutant clone lacking the *arp* gene locus (ΔArp) was generated. To achieve this, a telomere-mediated targeted-deletion strategy was utilized (Fig. 1A) (33–35). Briefly, this approach involves the introduction of a replicated telomere (*rtel*) at any position within a linear plasmid via integration of a deletion construct containing a target site for homologous recombination. Following integration of the deletion construct, the internal *rtel* is recognized and processed by the endogenous *B. burgdorferi* protein ResT (36, 37). Telomere resolution by ResT leads to the production of a new covalently closed hairpin end, resulting in deletion of the entire plasmid region located upstream or downstream of the target sequence.

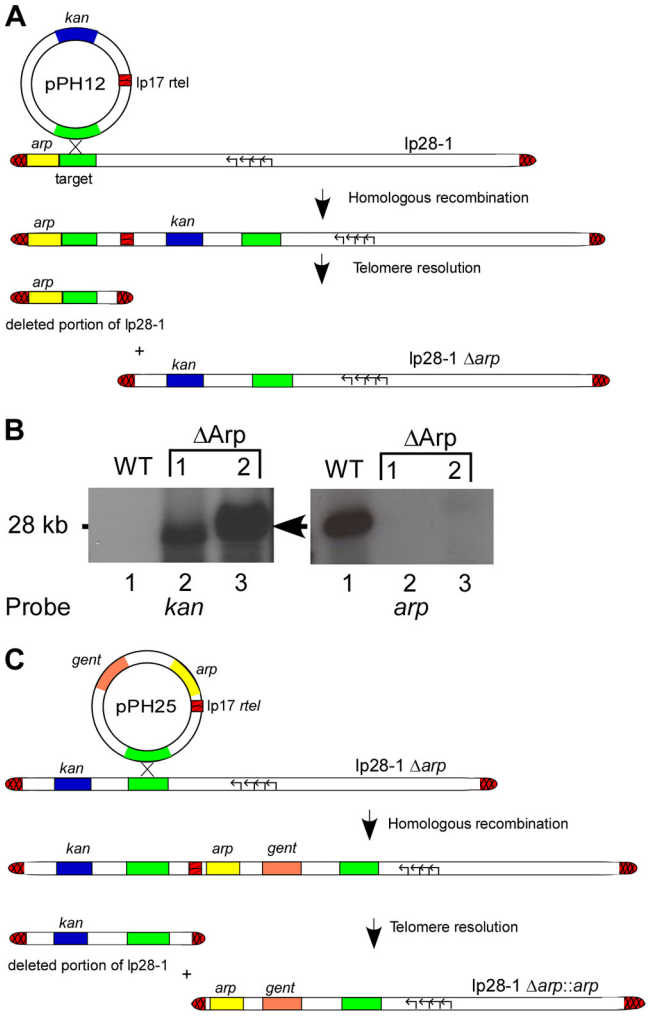

FIG 1 Generation of *arp* mutant clones. (A) Schematic of the deletion strategy for the *arp* knockout on lp28-1. The deletion construct (pPH12) was created by cloning a target region upstream of the *arp* locus (green) into a vector that carries a kanamycin resistance gene (*kan*) (blue) for selection and a replicated telomere (*rtel*) (red crosshatched regions) that is specifically recognized by the telomere resolvase, ResT. After transformation of the deletion construct into a fully infectious clone of *B. burgdorferi*, integration of the plasmid at the homologous target site and resolution of the *rtel* by endogenous ResT results in the loss of a DNA fragment containing the *arp* locus. (B) Southern blot confirmation of *B. burgdorferi* ΔArp mutant clones. Two clones matching the required criteria were selected for Southern blot analysis (lanes 2 and 3). The blot confirmed the presence of *kan* (arrow) and the absence of *arp* by the use of the respective probes on lp28-1. (C) Schematic of the complementation strategy of *arp* onto lp28-1Δ*arp*. To generate the *arp* replacement construct, the *arp* locus with the native promoter (yellow) was cloned into pPH12 to yield pPH25, which contained the same DNA target sequence homologous to lp28-1 that was used for targeted deletion of *arp*, along with a gentamicin resistance marker (orange) and an *rtel*. The complementation plasmid was then used to transform ΔArp competent cells.

As shown in Fig. 1A, *arp* is the first open reading frame (ORF) residing on lp28-1 and thus is the only lp28-1-resident gene lost following telomere resolution of the internally introduced *rtel*. Transformants were PCR screened for the presence of the kanamycin resistance cassette. Three clones meeting this criterion were further analyzed for their total plasmid profile, and two of the

TABLE 3 Infectivity of *B. burgdorferi* clones in C3H mice

Clone	No. of mice positive[a] at wk postinfection:										
								8			
	1 (blood)	2 (ear)	3 (ear)	4 (ear)	5 (ear)	6 (ear)	7 (ear)	Heart	Ear	Joint	Bladder
WT	5/5[b]	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
ΔArp	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
cArp	5/5	3/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

[a] Number of positive cultures/number of mice tested. Five mice were used for each *B. burgdorferi* clone.

three clones contained all of the parental plasmids. DNA isolated from these two mutant *B. burgdorferi* clones was then subjected to Southern blot analysis to confirm the loss of *arp* (Fig. 1B).

In order to verify that any potential reduction in joint swelling exhibited by the ΔArp mutant clone was due solely to the loss of the *arp* gene, a complemented Arp mutant clone was generated. The strategy for this involved reconstituting the *arp* gene back onto the lp28-1 plasmid harboring the *arp* deletion (Fig. 1C) (38). Previous studies have shown that transcriptional regulation of some *B. burgdorferi* genes is dependent on the topology (i.e., linear or circular) of the encoding genetic element (39). In addition, placement of *arp* back onto its native lp28-1 ensures the proper gene copy number (due to the lp28-1 copy number maintained in the cell), which can also have an effect on total protein expression.

To generate the *arp* replacement construct, the *arp* locus with the native promoter was cloned into the plasmid pPH25, which contained the same DNA target sequence homologous to lp28-1 that was used for targeted deletion of *arp*, along with a gentamicin resistance marker and *rtel* (Fig. 1C). Plasmid DNA was then transformed into ΔArp competent cells, and transformants were screened for the presence of the gentamicin and *arp* genes, as well as for all of the parental plasmids. One in *cis* Arp complement clone (cArp) that met all of the criteria was chosen for Western blot analysis in an attempt to ensure expression and surface localization of Arp. However, similar to previous findings with wild-type *B. burgdorferi* (40), expression of Arp could not be detected from any of the *in vitro*-derived *B. burgdorferi* clones (data not shown).

To assess the individual infectivities of the newly generated ΔArp and complemented *B. burgdorferi* clones, groups of 5 C3H/HeN (C3H) mice each were needle inoculated with the ΔArp, cArp, or wild-type *B. burgdorferi* clone. As shown in Table 3, blood samples collected at day 7 postinfection produced cultures positive for spirochetes in all inoculated mouse groups. Moreover, ear biopsy specimens collected at weeks 2 to 7 postinfection were positive for spirochetes in all mouse groups tested. Finally, ear, heart, bladder, and joint tissues harvested from sacrificed mice at week 8 postinfection produced cultures positive for spirochetes, demonstrating that the ΔArp and Arp complemented clones were fully capable of persistent host infection and dissemination.

Arp is required for early-onset joint swelling. Ankle swelling has been shown to reflect the amount of edema and to provide a gross measurement of the inflammatory response (31). To assess the requirement for Arp in ankle joint swelling, 5 C3H mice each were infected with either ΔArp, cArp, or wild-type *B. burgdorferi* clones. The progress of infection was monitored weekly via blood culture or ear biopsy samples. Gross ankle swelling was evaluated weekly by digital caliper measurements of the tibiotarsal joint thickness for up to 8 weeks postinfection. No significant differences were noted by day 7 postinfection ($P = 0.305$). Joint swelling in mice infected with the wild type was apparent beginning at 2 weeks postinfection and peaked between weeks 4 and 5 before starting to resolve (Fig. 2). Mice infected with the ΔArp clone exhibited drastically reduced joint swelling that was not statistically different from that in the uninfected-medium control mice throughout the duration of the study, except at the 8-week time point ($P = 0.037$). In contrast, the cArp complement clone group exhibited significantly greater swelling than the uninfected control group throughout the study period. No statistically significant difference was observed in joint swelling between cArp and the wild type, and both clones caused significantly higher swelling than the ΔArp clone ($P < 0.05$).

Overall, the above-described experiments show that mice infected with the ΔArp clone had reduced joint swelling during the first 5 weeks of infection and began to display increased swelling at week 6 postinfection. These results suggest that Arp is required for maximal severity of joint swelling during murine infection. Moreover, the pattern of joint swelling attributed to the ΔArp clone from the 8-week infection study suggests that Arp is specifically important for early-onset joint swelling.

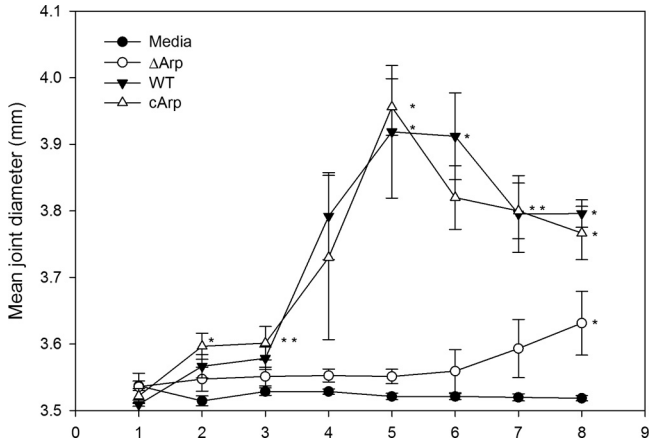

FIG 2 Tibiotarsal joint swelling in C3H mice infected with *B. burgdorferi* wild-type or *arp* mutant clones. Five mice were used in each group. The joints were measured with a digital metric caliper at weekly intervals after infection. The values are shown as the differences from uninfected, week zero measurements, and the mean joint diameter for each group is indicated at various weeks after infection. The error bars indicate the standard errors of the mean within the groups. An asterisk indicates a group(s) that was significantly different from one or more groups at a P value of <0.05. The joint swelling caused by infection with the ΔArp clone was not statistically different from that in the uninfected-medium control during the study period, except at week 8 ($P = 0.037$). There was no significant difference in joint swelling between the cArp- and wild type-infected mice throughout the study period, and both clones caused significantly more swelling than the knockout ($P < 0.05$).

TABLE 4 Histopathology scores of tibiotarsal joints

B. burgdorferi clone	Histopathology score (±SD) at wk[a]:		
	2	4	8
WT	3.3 ± 0.5	3.6 ± 0.55	2.2 ± 0.84
ΔArp	1.5 ± 1.0[b]	2.6 ± 1.14	2.4 ± 1.14
cArp	3.3 ± 1.0	1.2 ± 0.45[b]	1.6 ± 0.55

[a] Four mice were used for each *B. burgdorferi* clone at 2 weeks and 5 mice were used for each clone at weeks 4 and 8.
[b] Significantly different group using one-way ANOVA.

The presence of Arp contributes to efficient immune cell infiltration into the joint during early infection. Although the results from the above-described infection assays demonstrate the importance of Arp for maximal joint swelling caused by *B. burgdorferi* during the acute stages of host infection, it was unclear whether a decrease in joint swelling correlated with a reduction in joint tissue pathology. Caliper measurements of ankle swelling have been used to provide a gross measurement of the inflammatory response without the need to sacrifice the animal (41). However, they may correlate poorly with the severity of the underlying arthritis. Histological analysis provides a more complete determination of arthritis severity and characterization of the underlying inflammatory response (31). Our previous infection assay demonstrated that joint swelling levels in mice infected with the ΔArp clone started increasing notably at 6 weeks postinfection. Thus, it is conceivable that a difference in pathology between the ΔArp and wild-type clones could be clearly detected in joint tissues harvested at earlier time points.

To determine histopathological changes during infection, groups of C3H mice were infected with either ΔArp, cArp, or wild-type *B. burgdorferi* clones and sacrificed at 2, 4, or 8 weeks postinfection. Significantly reduced pathology was observed in mice infected with the ΔArp mutant compared to those infected with the wild type (scores, 1.5 ± 1.0 and 3.3 ± 0.5, respectively; $P = 0.034$) (Table 4) at 2 weeks postinfection. Mice infected with the cArp clone (score, 3.3 ± 1.0) showed severe joint pathology indistinguishable from that with wild-type *B. burgdorferi* (score, 3.3 ± 0.5) (Table 4). The tibiotarsal joints of mice infected with the wild-type and cArp clones displayed inflammation that was mainly characterized by infiltration of neutrophils involving the tibiotarsal flexor tendon and sheath (Fig. 3B and E, and C and F, respectively). Synovial hyperplasia and inflammation were also observed, and there was neutrophilic exudate in the lumen. There

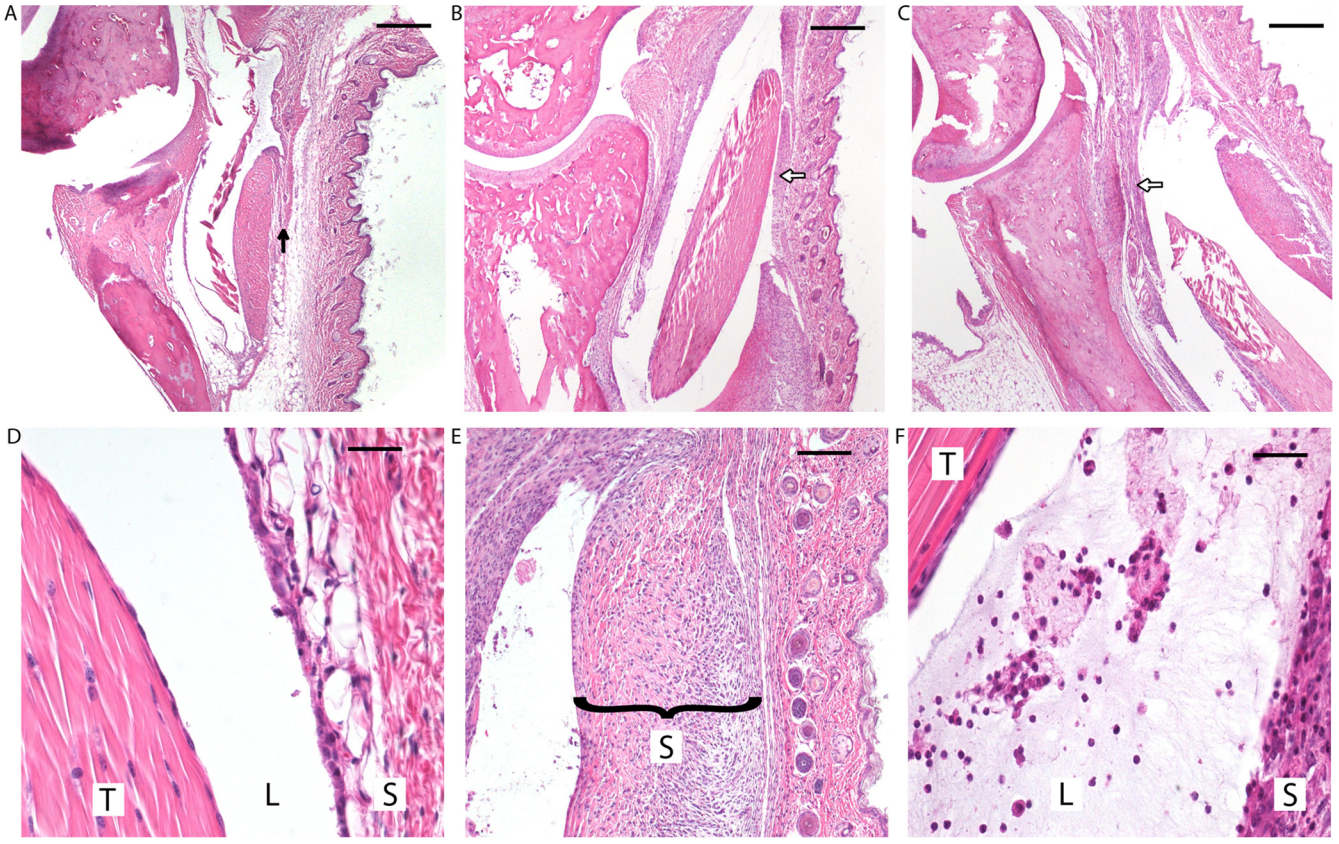

FIG 3 (Top row) Differences in tibiotarsal changes in C3H mice infected with *B. burgdorferi* clones at 2 weeks postinfection. (A) ΔArp. (B) Wild type. (C) cArp. Reduced cellular infiltration and synovial hyperplasia (black arrow versus white arrows) and minimal to absent neutrophilic infiltration were observed in the tibiotarsal flexor tendons of mice infected with the ΔArp clone. Histopathological changes between the WT and cArp clones were indistinguishable. (Bottom) High-power magnification of histopathologic changes in C3H mice infected with *B. burgdorferi* clones. (D) Minimal changes observed in the tibiotarsal flexor tendon (T) of ΔArp clone-infected mice with markedly reduced synovial hyperplasia and little to no neutrophilic infiltration in the lumen (L) of the tendon sheath (S) (magnification, ×100). (E) Inflamed hyperplastic tendon sheath in a mouse infected with wild-type *B. burgdorferi* (magnification, ×60). (F) Synovial lumen with inflammatory exudate comprised of neutrophils, edema residue, and fibrin and hyperplasia of the tendon sheath observed in mice infected with the cArp clone (magnification, ×100).

TABLE 5 *B. burgdorferi* quantification in tibiotarsal joints

B. burgdorferi clone[a]	No. of spirochetes/10^6 host cells (±SD) at wk:		
	2	4	8
WT	151 ± 113	2,033 ± 4,165	1,470 ± 1,188
ΔArp	206 ± 285	6,233 ± 4986[b]	2,167 ± 1,772
cArp	62 ± 87	6,569 ± 7,343	1,521 ± 1,859

[a] Five mice were used for each *B. burgdorferi* clone.
[b] Significantly different group using one-way ANOVA.

was also increased interstitial clear space and flocculent eosinophilic edema residue. Though this was recognized in some sections, it was difficult to quantitate histologically. All of these features, especially neutrophilic infiltration, were reduced in mice infected with the ΔArp clone (Fig. 3A and D).

In contrast to the week 2 histologic samples, increasingly similar levels of inflammatory-cell infiltration were observed in week 4 and 8 joint tissues infected with either the wild-type (scores, 3.6 ± 0.5 and 2.6 ± 1.14, respectively; $P = 0.064$) or the ΔArp (scores, 2.2 ± 0.84 and 2.4 ± 1.14, respectively; $P = 0.760$) *B. burgdorferi* clone (Table 4). Inflammation in both groups was characterized by infiltration of mainly neutrophils, macrophages, and lymphocytes. At 8 weeks, neutrophils were present but were not the predominant cell type. Joint tissues recovered from mice infected with the wild-type and ΔArp clones showed intermediate arthritis at week 8. The reduction in cellular infiltrate indicates early stages of arthritis resolution (9). ΔArp clone-infected mice showed intermediate levels of inflammatory-cell infiltration despite the low levels of ankle edema observed for mice infected with the mutant clone at 2 and 4 weeks postinfection (Fig. 2). Mice infected with cArp had reduced arthritis scores at weeks 4 and 8 (scores, 1.2 ± 0.45 and 1.6 ± 0.55, respectively). At 8 weeks, no significant difference in arthritis scores was observed between the ΔArp, wild-type, and cArp *B. burgdorferi* clones ($P = 0.355$). These findings suggest that the joint edema associated with the presence of Arp in *B. burgdorferi* aids in the efficient recruitment of inflammatory cells to the site of spirochete colonization during early-onset Lyme arthritis but is dispensable in the later courses of inflammation.

Arp contributes to control of the spirochete burden in joint tissues during infection. Following observations that deletion of *arp* reduced joint swelling and immune cell infiltration during early infection, we went on to determine if there would be an effect on spirochete colonization of the joint. To assess the spirochete burden in joints, qPCR analysis was conducted on collected joint tissues of C3H mice infected with either wild-type, ΔArp, or cArp *B. burgdorferi* clones at 2, 4, and 8 weeks postinfection. These time points were chosen because they represent early, peak, and resolving pathology based on results from the previous joint swelling experiments. At 2 weeks postinfection, there was no significant difference in spirochete burdens among the different *B. burgdorferi*-inoculated groups of mice ($P = 0.532$) (Table 5). After 4 weeks of infection, the number of spirochetes in the joints of mice infected with the wild-type clone was significantly lower than that in mice infected with the ΔArp clone (6.23×10^3 versus 2.03×10^3; $P = 0.027$). Next, it was determined whether the observed change in cellular infiltrate through time and resolution of arthritis corresponded to a decrease in the spirochete burden during infection with the experimental clones at 8 weeks postinfection. A reduction in the spirochete burden was observed in joints from mice infected with all three clones (Table 5). Though there was no statistically significant difference, mice infected with ΔArp consistently had higher numbers of spirochetes than mice infected with the wild type. Spirochete numbers from the joints of mice infected with the cArp clone compared to those infected with the ΔArp clone also showed no statistical difference at selected time points. From these results, the presence of Arp seems to equate to a generally lower burden of spirochetes in the joint, while the absence of Arp results in an overall increased spirochete burden in the joints. The results from the complement clone may hint that proper regulation of Arp expression in the clone is lacking, which could explain the high spirochete burden in the joints of mice infected with the clone at 4 weeks. Overall, the above-mentioned results suggest that the presence of the *arp* gene is associated with decreased spirochete burdens and increased pathology within the joint, and these phenotypes are more pronounced during early infection.

DISCUSSION

Studies with the murine model of Lyme disease have provided evidence that joint inflammation is a result of interaction of host immune responses with spirochete constituents (15–20). *B. burgdorferi* spirochetes are highly invasive and possess several lipoproteins that have been shown to have potent proinflammatory properties. These lipoproteins are encoded by genes that are regulated by diverse metabolic and immune microenvironments within mammalian tissues (21–24). The joint is thought to provide a protective niche for *B. burgdorferi* colonization, which may partially explain spirochete persistence in the tissue (42). In this study, we set out to determine if deletion of the *arp* gene has an effect on the pathogenesis of Lyme arthritis. The results reported here show a significant reduction in joint swelling in ΔArp clone-infected mice compared to those infected with wild-type *B. burgdorferi*, but only during the early onset of arthritis. The difference in the grossly measurable ankle swelling observed between the Arp mutant and wild-type *B. burgdorferi* during early host infection strongly suggests that Arp is required for the periarticular edema and inflammation associated with Lyme arthritis. We also observed that in the absence of *arp*, the spirochete load within the joint tissue remained high, suggesting that Arp expression may be somewhat detrimental to spirochete survival in that tissue.

The presence of Arp affects influx of inflammatory cells into joint tissue during early stages of infection. Although substantial reduction in joint swelling was observed, analysis of histopathology data on the joints of mice infected with the ΔArp clone at 4 and 8 weeks postinfection did not show any significant reduction in arthritis severity compared to those infected with the wild type. However, a significant difference in histopathology was observed when mice infected with the wild type (score, 3.3 ± 0.5) were compared to mice infected with the ΔArp clone (score, 1.5 ± 1.0; $P = 0.034$) at 2 weeks postinfection (Fig. 3), highlighting the importance of the lipoprotein in the early stages of infection. The edema associated with ankle swelling is typically correlated with infiltration of neutrophils and macrophages in response to the presence of spirochetes in the subcutaneous tissue (9). Our results suggest that the significant reduction of joint swelling observed after infection with ΔArp corresponds to decreased influx of inflammatory cells into joint tissue during early murine infection with *B. burgdorferi*. Several inflammatory cytokines and chemokines have been shown to influence the infiltration of neutrophils into *B. burgdorferi*-infected joint tissue (43, 44). Recently, BBA57

was shown to be a major trigger of Lyme arthritis, with its deficiency resulting in decreased neutrophil chemotaxis (45). The results of this study suggest that Arp may stimulate recruitment of neutrophils or other immune components, allowing the production of cytokines that lead to periarticular edema. Further studies will be needed in order to determine if the cytokine profile differs between mice infected with wild-type *B. burgdorferi* and those infected with the Arp deletion mutant.

In agreement with the findings reported here, a study by Imai et al. published during the preparation of the manuscript showed joint arthritis to be significantly reduced in C3H mice infected with an *arp* mutant clone at day 14 postinfection (26). However, the Imai et al. study also observed that their *B. burgdorferi arp* mutant displayed reduced joint pathology for the entire 42-day infection period, which contrasts with the present study, which found a significant difference in pathology only at day 14. Although mouse experiments in both studies involved an infectious-dose inoculum of 10^5 spirochetes or higher, it is possible that the ΔArp clone used here, if diluted, may have demonstrated an attenuated phenotype relative to the wild type at later time periods. Several additional factors might also explain the discrepancies between the two studies. First, the methods of gene deletion utilized differed between the two studies (allelic exchange [26] versus telomere-mediated deletion [this study]). It is possible that differences in these methodologies could have resulted in subtle genetic alterations or polar effects on flanking genes. A second factor is that complementation typically involves reintroduction of a wild-type copy of only the gene of interest (46). However, the Imai et al. study complemented *arp* through displacement of the altered lp28-1 plasmid via transformation with a wild-type version of lp28-1, which has the potential to mask any additional (non-*arp*-related) genetic changes that might have contributed to the observed phenotype (26). Finally, this study set out to specifically characterize how joint cell infiltration and edema were affected by the deletion of *arp*, which was not explicitly analyzed in the recent publication. Our results show that Arp contributes greatly to the generation of neutrophilic inflammation and joint edema during the early phase of infection, as shown by joint swelling experiments. This suggests that Arp may play a role in cytokine changes responsible for recruitment of neutrophils to the *B. burgdorferi*-colonized joint tissue and subsequent edema. Elucidating the mechanisms, cells, and cytokines involved will require further investigation.

Arp is immunopotent and may contribute to control of the spirochete burden in the joint. Pathology during *B. burgdorferi* infection has been primarily associated with host inflammatory responses (11, 19), and accordingly, invasion of joint tissues by *B. burgdorferi* has been linked with the pathogenesis of arthritis (46–48). Arp has been shown to be highly immunogenic, and antibody against the lipoprotein has been detected from day 7 postinfection up to day 90 (25). In this study, deletion of *arp* resulted in a burden of spirochetes within joint tissues at 4 weeks postinfection significantly higher than that exhibited in mice infected by the wild type (6.23×10^3 versus 2.03×10^3, respectively; $P = 0.027$) and was comparatively high at additional time points. Immune recognition of Arp in mice infected by wild-type *B. burgdorferi* may explain the observed decrease in histopathology scores obtained at early and peak infection compared to late infection (scores, 3.3 ± 0.5 at 2 weeks and 3.6 ± 0.5 at 4 weeks compared to 2.2 ± 0.84 at week 8; $P = 0.111$ and $P = 0.014$, respectively). No statistically significant differences were noted in the histopathology scores of mice infected with the ΔArp clone at similar time points. This suggests that expression of Arp may be detrimental to *B. burgdorferi* survival, which may hint at an underlying important biological role for the lipoprotein during host infection. Moreover, the finding that ΔArp spirochetes are present at higher numbers in joint tissues suggests that Arp may not be involved in the tropism of *B. burgdorferi* to this tissue site. In addition, there was no statistically significant difference between the spirochete burdens in the joints of mice infected with cArp and ΔArp at week 4, despite the measured difference in tibiotarsal swelling at this time point and beyond. This raises the question of whether the difference in spirochete numbers at week 4 between the wild type and the ΔArp clone is biologically significant. It would also argue that arthritis is not directly correlated with bacterial levels and suggests that there is no fitness defect associated with the loss of *arp*.

Again, contrary to the findings reported here, the recently published study by Imai et al. (26) found that the spirochete burden was lower in mice infected with the *arp* mutant clone. This may be due to the observed impaired dissemination reported by the authors, which we did not observe in this study. A possible explanation for this discrepancy is that genetic manipulation of lp28-1 in the Imai et al. study may have had an effect on a yet to be identified gene residing on the plasmid that has been suggested to express a putative regulator of the outer surface protein OspC (49). It has been recently shown that OspC may be an important dissemination factor of *B. burgdorferi* during mammalian infection (50). Once again, because the method of complementation reported in the Imai et al. study involved total plasmid replacement, any potential genetic alterations of lp28-1 affecting OspC regulation that may have led to impaired dissemination by the *arp* mutant could have been masked in the complemented clone.

Overall, the findings presented here demonstrate the requirement for Arp in *B. burgdorferi*-induced joint swelling and pathology. Further studies directed toward elucidating the specific role that Arp has in pathogenesis and, possibly, immune manipulation by the Lyme disease spirochete will likely provide insight into the mechanisms responsible for *B. burgdorferi*-associated joint inflammation and aid in the understanding of Lyme disease pathogenesis.

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