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SpyA, a C3-Like ADP-Ribosyltransferase, Contributes to Virulence in a Mouse Subcutaneous Model of *Streptococcus pyogenes* Infection▽†‡

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Streptococcus pyogenes **is an important human pathogen with an expansive repertoire of verified and putative virulence factors. Here we demonstrate that a mutant deficient in the production of the streptococcal ADP-ribosyltransferase SpyA generates lesions of reduced size in a subcutaneous mouse infection model. At early stages of infection, when the difference in lesion size is first established, inflamed tissue isolated from lesions of mice infected with *spyA* mutant bacteria has higher levels of mRNA encoding the chemokines CXCL1 and CCL2 than does tissue isolated from mice infected with wild-type bacteria. In addition, at these early times, the mRNA levels for the gene encoding the intermediate filament vimentin are higher in the mutant-infected tissue. As wound resolution progresses, mRNA levels of the gene encoding matrix metallopeptidase 2 are lower in mutant-infected tissue. Furthermore, we demonstrate that the *spyA* mutant is internalized more efficiently than wild-type bacteria by HeLa cells. We conclude that SpyA contributes to streptococcal pathogenesis in the mouse subcutaneous infection model. Our observations suggest that the presence of SpyA delays wound healing in this model.**

ADP-ribosyltransferases (ADPRTs) are enzymes that covalently attach the ADP-ribose moiety of NAD to a target protein. While modification is often reversible and is used to modulate cellular function in a number of bacteria and eukaryotes, some pathogenic bacteria employ ADPRTs for more sinister aims (reviewed in references 9, 14, and 32).

C3-family ADPRTs share a number of characteristics in common. They are produced by Gram-positive organisms, modify Rho GTPases, are approximately 25 kDa in size, and, unlike other bacterial ADPRTs with pathogenic potential, they lack an apparent translocation domain (61). Initially, it was unclear how the toxins would access their eukaryotic targets, and this raised questions concerning their importance for virulence. However, the mode of entry for several of the C3 enzymes has recently been elucidated. C3stau2 has been shown to directly enter the cytoplasm of cells that contain ingested *Staphylococcus aureus* (43). C3lim and C3bot1 were shown to specifically intoxicate monocytes and macrophages via a receptor-mediated process requiring acidification of endosomes (17).

Streptococcus pyogenes (group A *Streptococcus* [GAS]) is a ubiquitous human-adapted Gram-positive pathogen of considerable public health importance. Infections range in severity from minor tonsillitis and pharyngitis to life-threatening necrotizing fasciitis and streptococcal toxic shock syndrome (11). An increase in the incidence of invasive infections beginning in the latter part of the 20th century has resulted in increased research into and identification of the organism's numerous virulence determinants. Though typically considered an extracellular pathogen, GAS have been shown to enter and survive intracellularly in macrophages (60), neutrophils (40, 41), and epithelial cells (24, 30, 34, 38, 42, 54).

Recently, *S. pyogenes* was shown to transcribe a gene encoding a C3-like ADPRT, *spyA*, during mid-log growth in rich medium (10). The authors further demonstrated that recombinant SpyA possesses both NAD-glycohydrolase and ADP-ribosyltransferase activity *in vitro* (10). Multiple studies have shown that *spyA* transcripts are abundant during mouse soft tissue infection, growth in human blood, and growth in human saliva (21, 22, 55), and it has been shown that *spyA* is more highly transcribed during invasive infection than during pharyngeal infection (59). Additionally, at least two regulatory systems that play a role in GAS virulence, CovR/S and Ihk/Irr, have been shown to have an effect on *spyA* transcription (20, 62).

Expression of SpyA resulted in the loss of stress fibers in transfected cells; however, unlike other C3-ADPRTs, which mediate actin rearrangements through modification of small Rho GTPases, SpyA directly modified actin filaments (10). SpyA was also shown to modify several proteins other than actin, including the intermediate filament vimentin (10). Vimentin participates in many cellular processes, including wound healing (15, 19, 25) and immune function (5, 26, 52).

We have employed a mouse model of subcutaneous infection in conjunction with a tissue culture infection model to investigate the role of SpyA in GAS pathogenesis. We found that a mutant deficient in SpyA expression produces cutaneous

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‡ Dedicated to the memory of our mentor, colleague, and dear friend, Carleen M. Collins, 1955-2008.

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lesions of reduced size and is associated with increased transcription of *Cxcl1*, *Ccl2*, and *Vim* and decreased transcription of *Mmp2* in infected tissue, all factors with roles in wound healing. In a tissue culture model, the *spyA* mutant is internalized more efficiently than wild-type bacteria by HeLa human epithelial cells.

MATERIALS AND METHODS

Bacteria and tissue culture. *Streptococcus pyogenes* strain MGAS5005 (wild type; ATCC BAA-947) is an M1-serotype strain isolated from the cerebral spinal fluid of a patient with bacterial meningitis (58) and was generously provided by J. M. Musser. *Escherichia coli* strains DH5α (23) and GM119 (3) were used as intermediate plasmid hosts during cloning. *S. pyogenes* cultures were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) or on THY supplemented with 1.5% Bacto agar (BD Biosciences, San Jose, CA). Cultures were grown without aeration at 37°C. Prior to animal or tissue culture infection, GAS cultures were grown standing at 37°C in a 5% CO_2 atmosphere. *E. coli* was grown in Luria-Bertani (LB) medium or on LB agar. When required, antibiotics were included at the following concentrations: erythromycin at 1 μg/ml for GAS or 200 μg/ml for *E. coli* and carbenicillin at 100 μg/ml for *E. coli*. Mid-log phase was determined spectrophotometrically as an optical density at 600 nm (OD_{600}) of approximately 0.35 for *S. pyogenes*.

HeLa cells (ATCC CCL-2) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 in a humidified chamber. All cell culture medium was obtained from GIBCO/Invitrogen (Carlsbad, CA) unless otherwise noted.

DNA techniques. Genomic DNA was isolated from crude lysates of overnight GAS cultures using the DNeasy tissue kit (Qiagen, Valencia, CA). Plasmid DNA was isolated from *E. coli* using alkaline lysis followed by ethanol precipitation for large-scale preparations or the FastPlasmid Mini kit (Eppendorf) for small quantities. Plasmid purification from strain GM119 using the FastPlasmid Mini kit included an additional wash step using 1 M guanidine-HCl in 50% isopropanol. PCR was carried out using either GoTaq (Promega, Madison, WI) or *Pfu* Turbo polymerase (Stratagene), and sequencing was performed using the BigDye Ferminator, version 3.1 (Applied Biosystems).

Construction of MGAS5005 *spyA*. PCR was used to amplify *spyA* and surrounding DNA from the MGAS5005 genome with the primers KOspyAF (GA GATCTAGATATGTCGAGTGAGGCAAGACG) and KOspyAR (GAGACC CGGGTGAGAAACTGGTGGTGTCAAGAGC), which introduce XbaI and XmaI sites, respectively (underlined bases). The resulting 1,157-bp amplicon was inserted into the cloning vector pGEM-T Easy (Promega) as per the manufacturer's instructions to generate the plasmid pGEMspyAFlank. A second PCR was performed using the primers KOSpyAIOF (GAGCTCACCTTTGGCTTT GTCAGGCCTTAGGCTGGTGTTGATAGG) and KOSpyAIOR (CCTTCAG AGGTTGAGCTCAGGCCTTTGTTTCCAAGAGGCTGTC) to introduce StuI restriction sites (underlined bases). The product was digested with StuI (New England BioLabs, Ipswich, MA) and ligated to generate the desired deletion: 378 bp were removed, and 6 were introduced as a restriction site for cloning. The resulting 791-bp *spyA*Δ fragment was excised using XbaI and XmaI (NEB) and inserted into the temperature-sensitive vector pJRS233 (49), provided by J. R. Scott, to create the plasmid p*spyA*Δ. The plasmid was introduced into MGAS5005 by electroporation, after which we used a multistep approach to obtain a deletion mutant. Briefly, transformants were serially grown in the presence of antibiotics at 37°C, the nonpermissive temperature, to promote integration. Single crossover mutants were identified and subsequently serially grown without antibiotics at 30°C, the permissive temperature, to promote plasmid excision and loss. The *spyA* deletion was verified by PCR, sequencing, and reverse transcription-PCR (RT-PCR).

Western analysis of secreted proteins. Wild-type and *spyA* mutant bacteria were grown to both stationary and mid-log phases of growth in THY. Bacteria were pelleted, and the supernatant was passed through a 0.22-μm polyethersulfone filter (Millipore). Supernatants were then concentrated in an Amicon Ultra-15 centrifugation device with a molecular weight cutoff 10 kDa. Concentrated supernatants were normalized to the optical density at the time of harvest, and equal amounts were separated on 4 to 20% gradient gels, transferred to nitrocellulose, and blocked with Tris-buffered saline (pH 7.4) (TBS)–1% Tween containing 5% milk. Blots were probed for secreted proteins using anti-SpeA (31), anti-Mac/IdeS (a generous gift from B. Lei, Montana State University—Bozeman), and antistreptokinase (US Biologicals) antibodies, followed by the appropriate secondary antibody conjugated to horseradish peroxidase (HRP). Detection was performed using the enhanced chemiluminescence (ECL) (Pierce) or ECL Plus (Amersham Biosciences) kit, following the manufacturer's instructions.

Mouse infections. All studies conformed to NIH guidelines and were approved by the Animal Care and Use Committee at Montana State University. Female Crl:SKH1-*Hr*^{hr} mice (Charles River Laboratories) were used for all subcutaneous infections. This outbred line is hairless and immunocompetent (53) and is a well-established model for GAS subcutaneous infection (8, 22, 39, 57, 68). Mice were given food and water *ad libitum*.

Mid-log-phase bacteria were pelleted and washed 2 times with cold phosphate-buffered saline (PBS) (Invitrogen). Bacteria were resuspended to the desired concentration in PBS and kept on ice until use. Mice were infected subcutaneously with ~2×10^7 bacteria (15 mice per strain) in 50 μl of PBS or with PBS alone (10 mice). The resulting lesions were measured daily for 14 days, and the area was calculated using the formula for an ellipse (½ length × ½ width × π). Two mice infected with the *spyA* mutant bacteria were excluded from analysis. One animal had a lesion located on its side, rather than the back; another animal had an anomalous lesion, indicating that the bacteria had been introduced intradermally rather than subcutaneously.

Animals for histological examination of lesions or RNA isolation from tissue were infected with ~8×10^6 wild-type or *spyA* mutant bacteria administered in 50 μl PBS. Three mice from each group were sacrificed at 12 h postinfection, and the developing lesions were excised and formalin fixed. At 24, 48, and 72 h, six mice from each group were sacrificed and the infected tissue was removed. Three tissue specimens from each group were formalin fixed, and the remaining three were homogenized in 2 ml PBS, serially diluted, and plated. After plating, the homogenate was pelleted, resuspended in buffer RLT (Qiagen), and frozen at −80°C until RNA extraction. Uninflamed tissue from infected mice was also obtained for comparison.

Tissue staining. Formalin-fixed tissues were trimmed, embedded in paraffin, sectioned, and hematoxylin and eosin (H&E) or Gram stained at Idexx Laboratories, Preclinical Research Services (West Sacramento, CA) as per their standard protocols. Sections were scored for inflammation by trained Idexx pathologists.

RNA extraction and transcriptional analysis. Mouse tissue that had been disrupted for quantification of bacteria and frozen in buffer RLT was thawed, transferred to tubes containing Lysing Matrix D (MP Biosciences), and processed in a Mini-BeadBeater-8 instrument (Biospec Products) set to homogenize for 1.5 min. After cooling on ice for approximately 5 min, samples were processed for an additional 1.5 min. RNA was isolated from 100 μl of the homogenate (~30 mg tissue) by adding 200 μl buffer RLT and following the manufacturer's instructions for the RNeasy fibrous tissue kit (Qiagen). RNA was quantified, and transcription of the genes *Cxcl1*, *Ccl2*, *Il10*, *Il6*, *Tnf*, *Il1b*, *Ifng*, *Mmp2*, *Fgf2*, *Vim*, and *Gapdh* was analyzed using QuantiTect primer assays and the QuantiTect SYBR green RT-PCR kit (Qiagen) in a Corbett RotorGene 3000 thermal cycler as per the manufacturer's instructions. Transcript levels were determined using the relative standard curve method as described by Johnson et al. (29). *Gapdh* transcript levels were used to normalize mRNA levels between mice. Data are reported as the average fold change compared to mRNA levels at 24 h postinfection in wild-type-infected mice.

Neutrophil isolation. Heparinized venous blood samples from healthy donors were collected in accordance with protocols approved by the Institutional Review Boards for Human Subjects at Montana State University and the University of Washington. Neutrophils were isolated under endotoxin-free conditions (<25.0 pg/ml) as described previously (6, 65). Briefly, leukocytes were separated from the blood using dextran sedimentation, and polymorphonuclear leukocytes (PMNs) were further separated from monocytes using gradient centrifugation followed by lysis of erythrocytes. Cell viability and purity of preparations were assessed by flow cytometry (FACSCalibur; BD Biosciences). Cell preparations contained ~99% neutrophils.

Neutrophil assays. The ability of wild-type and *spyA* mutant bacteria to survive phagocytosis by neutrophils was assessed using synchronized phagocytosis as described previously (65) with the following modification: experiments were performed in 96-well plates. All values are reported as the percentages of bacteria obtained at time zero (T_0) by using the following formula: (CFU_{+PMN} at T_n/CFU_{+PMN} at T_0) × 100. Assays examining phagocytosis of GAS by PMNs were performed using fluorescence-activated cell sorter (FACS) analysis as previously described (63, 65).

Internalization into epithelial cells. Confluent monolayers of HeLa cells were incubated with wild-type or *spyA* mutant bacteria at a multiplicity of infection (MOI) of ~10:1 for in a 24-well dish. After 3 h, wells were washed 3 times with warmed PBS to remove nonadherent bacteria. One-half of the wells were lysed by the addition of ice-cold water, serially diluted, and plated on THY to determine the total number of cell-associated (adherent and internalized) bacteria.

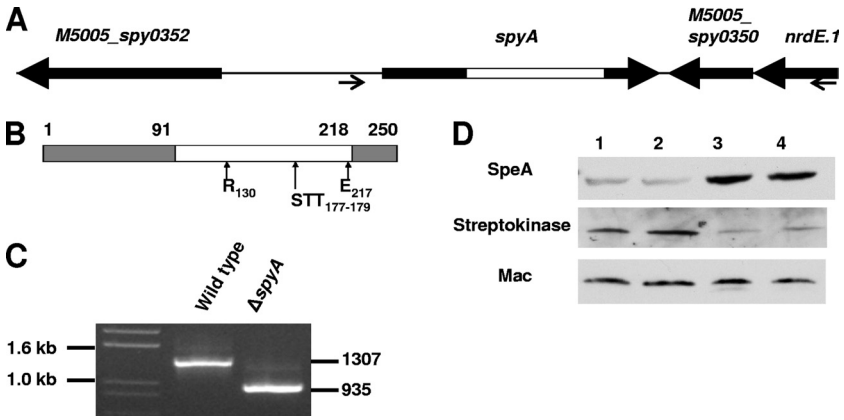

FIG. 1. Deletion of *spyA*. (A) Chromosomal location of *spyA*. The white bar indicates deleted region. Arrows below chromosome show locations of primers used for PCR analysis. (B) SpyA protein. Conserved residues required for ADP-ribosyltransferase activity are shown. The gray bar shows protein; the white area represents the deleted region. Numbers above protein indicate residues retained in the deletion construct. (C) PCR using primers shown in panel A. Size standards are shown on the left; sizes of PCR fragments are shown on the right. (D) Western blots to detect SpeA, streptokinase, and Mac in GAS supernatants. Lanes 1 and 2 are mid-log; lanes 3 and 4 are stationary phase. Lanes 1 and 3 are supernatants from the wild type, and lanes 2 and 4 are from the mutant.

The remaining wells were treated with DMEM plus 10% FBS containing 5 μg/ml penicillin G and 100 μg/ml gentamicin for an additional hour to kill any extracellular bacteria. After antibiotic treatment, wells were washed 3 times with warmed PBS, lysed with cold water, serially diluted, and plated to THY. Percent internalization is defined as (CFU after antibiotic treatment)/(CFU prior to antibiotic treatment) × 100.

Statistical analysis. All statistical analysis was done using the Prism software program (GraphPad, La Jolla, CA). Analyses comparing two groups were done using the Mann-Whitney U test or an F test to compare variance, followed by Student's *t* test. Three or more groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's posttest for pairwise comparisons.

RESULTS

Construction and characterization of a GAS mutant deficient in production of SpyA. An in-frame deletion in *spyA* was generated using an allelic exchange approach. The deletion removed 378 bp from within the gene, including the nucleotides encoding conserved residues known to be important for catalysis by all ADPRTs (the conserved arginine, Ser-Thr-Ser motif, and catalytic glutamate [10]) and introduced a 6-bp restriction site for cloning purposes (Fig. 1A and B). Although *spyA* appears to be encoded in a monocistronic operon (Fig. 1A), the deletion was constructed to preserve the coding frame, thereby minimizing any potential polar effects. The deletion was confirmed by PCR (Fig. 1C), sequencing, and RT-PCR. No difference in growth rate was observed between the mutant and wild-type bacteria in THY broth, nor were morphological differences apparent upon microscopic examination of Gram-stained bacteria. Attempts to complement the mutation were hampered by toxicity of active SpyA for *E. coli*, and ultimately complementation was unsuccessful.

Secretion of known virulence factors is not altered in the *spyA* mutant. Although the deletion is nonpolar and *spyA* lies in an operon by itself, a portion of the *spyA* sequence, including a putative secretion signal, remains in the chromosome of the mutant. To assess the possibility that a truncated protein may cause global secretion defects, we examined supernatants for the presence of SpeA, streptokinase, and Mac/IdeS. Western analysis was performed on equal volumes of supernatant from a minimum of 2 biological replicates in both exponential and stationary growth phases. Since the *spyA* mutant does not exhibit a growth defect, each strain was allowed equal opportunity to replicate and secrete proteins. Densitometric scanning of the resulting immunoblots did not detect a decrease in the amount of SpeA, Mac/IdeS, or streptokinase found in the supernatant. We concluded that the *spyA* deletion is unlikely to have resulted in global secretion defects in the mutant strain (Fig. 1D).

The *spyA* mutation significantly reduces lesion size but not bacterial burden during subcutaneous infection. Subcutaneous infection models have been used extensively to study the role of streptococcal genes in pathogenesis (22, 59, 62). We employed this approach to examine the role of SpyA in GAS infections. Since it has been shown that *spyA* is transcribed during mid-exponential growth (10), bacteria harvested at this stage were used to infect female Crl:SKH1-Hr^{hr} mice subcutaneously on the back. Mice received 2.2×10^7 wild-type bacteria, 2.3×10^7 *spyA* mutant bacteria, or sterile PBS, after which they were observed daily for 14 days. Control mice did not develop lesions after PBS injection. Lesions on mice infected with wild-type bacteria were larger on all days, with differences achieving statistical significance on days 2 to 5 and 7 to 11 (Fig. 2A, day 6, $P = 0.06$). By 24 h postinfection, all mice that received bacteria had developed a purulent lesion at the injection site. By day 2, the lesions on all but one mouse (*spyA* mutant infected) exhibited dermal necrosis, and by day 3 all lesions were necrotic. On day 2, the median lesion size for mice infected with wild-type bacteria had increased from that for day 1, whereas the median lesion size in the group infected with the *spyA* mutant had decreased. All mice survived the duration of the experiment, at which time most lesions were nearly resolved or were resolved, regardless of the infecting strain. Histological examination of tissues at early times postinfection showed marked infiltration of leukocytes, high numbers of bacteria, and central necrosis of the lesion similar to that seen by Graham et al. after 2.5 days of infection (22), but no

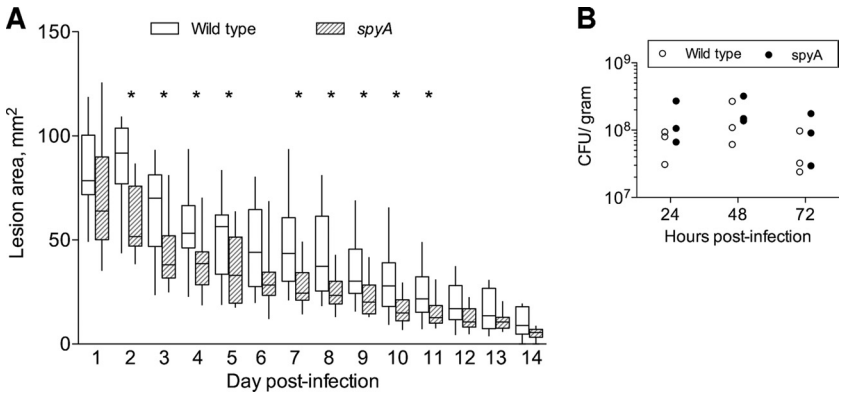

FIG. 2. Skin lesions formed by *S. pyogenes* in Crl:SKH1-*Hr*^{hr} mice. (A) Mice were infected subcutaneously with 2×10^7 bacteria, and resulting lesions were measured daily for 2 weeks ("*" indicates $P < 0.05$ using the Mann-Whitney test). The plot shows median, quartiles, minimum, and maximum. Clear boxes represent wild-type-infected mice ($n = 15$), and gray boxes represent *spyA* mutant-infected mice ($n = 13$). (B) CFU per gram of lesion tissue were determined at 24, 48, and 72 h postinfection in mice infected with 8×10^6 bacteria. No significant differences were detected using a two-tailed, unpaired Student *t* test after confirming equal variance by F test. Each circle represents a single mouse. Open circles represent wild-type CFU, and filled circles represent *spyA* CFU.

differences between wild-type-infected and *spyA* mutant-infected tissues were detected.

In a separate experiment, the influence of SpyA on bacterial burden was assessed. Bacterial counts were determined at 24, 48, and 72 h by serial dilution and plating of homogenized infected lesions. Replication occurred *in vivo*, and both strains reached a maximum of approximately 2×10^8 organisms per gram of tissue at 48 h postinfection (Fig. 2B). Differences between the number of wild-type and mutant bacteria in infected tissues were not significant at any time point examined (calculated using unpaired Student's *t* test after an F test to verify that variances were not significantly different). Similarly, histological examination of Gram-stained tissue sections revealed no differences in the number of bacteria present, nor did H&E-stained tissues reveal differences in the quality of the lesions based on the type or quantity of infiltrating leukocytes (not shown).

Transcript levels of *Cxcl1* and *Ccl2* are higher at early times postinfection in mice infected with *spyA* mutant bacteria. Since the difference in size between lesions caused by wild-type and *spyA* mutant bacteria was established early in infection without detectable differences in the composition of the lesions or in the number of bacteria present, we hypothesized that there was an early difference in induction of the innate immune response. To address this hypothesis, we investigated chemokine and cytokine production in the two groups of infected animals. Since CXCL1 and CCL2 are well-characterized neutrophil and monocyte chemoattractants (45, 67), we investigated mRNA levels of *Cxcl1* and *Ccl2* at 24 h postinfection. Tissues infected with the *spyA* mutant strain had increased transcript abundance versus those found in mice infected with wild-type bacteria. By 72 h, mRNA levels of *Cxcl1* and *Ccl2* had decreased in mice infected with *spyA* mutant bacteria, while levels in mice infected with wild-type bacteria did not significantly differ over this time period (Fig. 3), suggesting that *spyA* influences early innate immune responses associated with these factors.

In addition to *Cxcl1* and *Ccl2* transcription, we analyzed the transcription of other genes known to play a role in innate immunity, *Tnf*, *Il1b*, *Il6*, *Il10*, and *Ifng*. No significant differences between wild-type-infected and *spyA* mutant-infected tissues were detected (not shown). Levels of *Ifng* mRNA were below the limit of detection for all tissues examined (not shown).

Early postinfection levels of *Vim* mRNA are higher in *spyA* mutant-infected mice. Since histology did not reveal differences in leukocyte infiltration to infected sites but quantitative

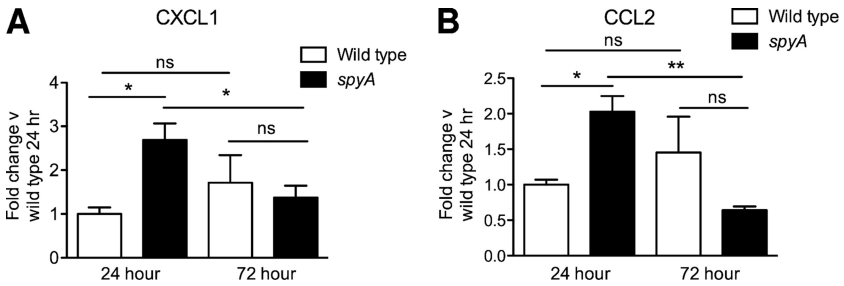

FIG. 3. *Cxcl1* and *Ccl2* expression in GAS-infected tissue. RNA was isolated from inflamed tissue at 24 and 72 h postinfection, and levels of *Cxcl1* (A) or *Ccl2* (B) transcripts were quantified in relation to that of *Gapdh* using qRT-PCR. Values reported are fold change compared to values for wild-type-infected tissue at 24 h. Graphs show means and SEM of results for 3 mice per group at each time, 2 to 3 technical replicates per mouse. ("*" indicates $P < 0.05$ by two-tailed, unpaired Student's *t* test; "**," $P < 0.01$). Clear bars represent mice infected with wild-type bacteria; black bars represent mice infected with *spyA* mutant bacteria.

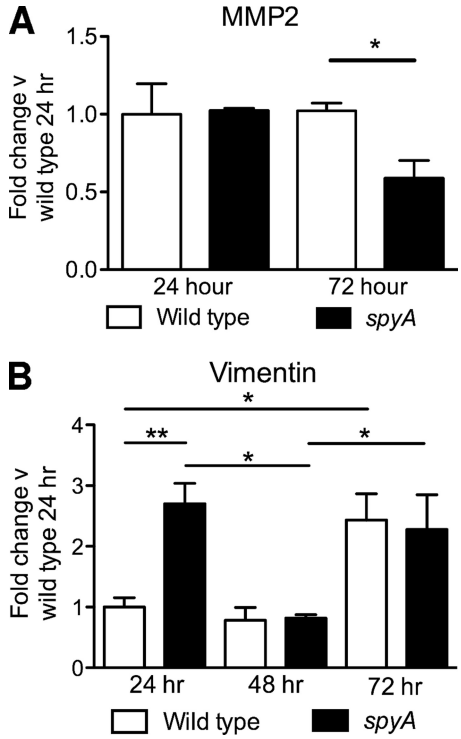

FIG. 4. Expression of *Mmp2* and *Vim* in infected tissue. RNA was isolated from inflamed tissue, and using qRT-PCR levels of *Mmp2* (A) or *Vim* (B), transcripts were determined in relation to *Gapdh* transcripts and normalized to levels found in wild-type-infected tissue at 24 h. Graphs show means and SEM of 3 mice per group (2 to 3 replicates per mouse) at each time. *, $P < 0.05$; **, $P < 0.01$. A two-tailed, unpaired Student's *t* test was used to compare the wild type to the mutant, and ANOVA followed by Tukey's posttest was used to compare across time points. White bars represent wild-type bacteria, and black bars represent *spyA* mutant bacteria.

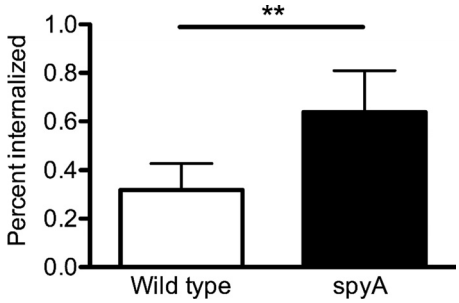

FIG. 5. Internalization of GAS into HeLa cells. Wild-type and *spyA* mutant bacteria were incubated with HeLa cells for 3 h, at which point associated bacteria were quantified, or cells were treated with antibiotics for 1 h. No difference was detected in associated bacteria at 3 h. The graph shows the percentage of associated bacteria that remain viable after antibiotic treatment. "**" indicates $P < 0.01$ using a two-tailed, paired Student *t* test. Bars show means and standard errors of results from 7 independent experiments.

RT-PCR (qRT-PCR) did reveal differences in transcription of *Cxcl1* and *Ccl2*, we hypothesized that functions of CXCL1 and CCL2 other than those on leukocyte recruitment may play a role in the formation and resolution of GAS subcutaneous lesions. In addition to their roles in phagocyte recruitment, CXCL1 and CCL2 have been demonstrated to play a role in wound healing (36, 51). To test the hypothesis that *spyA* influences wound healing, we examined the transcription of other genes involved in repair of cutaneous injury, specifically those encoding matrix metallopeptidase 2 (*Mmp2*), fibroblast growth factor 2 (*Fgf2*), and vimentin (*Vim*). MMP2 is a constitutively expressed protease capable of degrading the extracellular matrix and is upregulated after cutaneous injury (37). Fibroblast growth factor 2 (FGF2) is a small protein mitogenic for a number of cell types; mice lacking the gene display delayed wound healing (46). No difference was seen in the amount of *Mmp2* or *Fgf2* mRNA when comparing infection with wild-type and mutant bacteria at 24 h; however, at 72 h postinfection, *Mmp2* levels were lower in tissue infected with the mutant compared to that infected with the wild type (Fig. 4A). In contrast to *Mmp2* levels, *Vim* mRNA was significantly more abundant in mice infected with mutant bacteria than in those infected with wild-type bacteria at 24 h (Fig. 4B). Interestingly, by 48 h transcription had decreased to levels similar to those in the wild-type infections. This further supports the hypothesis that SpyA plays a role early during lesion formation by altering host responses.

Interaction of *spyA* mutant bacteria with neutrophils and epithelial cells. *S. pyogenes* evades killing by neutrophils by several well-defined mechanisms, including inhibition of phagocytosis, survival following phagocytosis, and neutrophil lysis (reviewed in reference 64). To determine if *spyA* contributes to this ability, we compared wild-type and *spyA* mutant bacteria during interaction with human neutrophils. Human neutrophils were isolated and incubated with opsonized bacteria for up to 5 h. Both strains were highly resistant to killing by PMNs, consistent with previous observations (65). Differences in survival after exposure to neutrophils between SpyA mutant and wild-type strains did not achieve statistical significance, nor were differences in association or phagocytosis observed between wild-type and *spyA* mutant bacteria (see Fig. S1 in the supplemental material). Assays measuring the amount of lactate dehydrogenase released from infected neutrophils showed that while both strains were proficient at killing neutrophils, there was no difference in the cytotoxicity of the two strains at times up to 5 h (not shown).

Since we did not see a significant effect on the primary immune cells we tested, we hypothesized that *spyA* may alter bacterium-host interactions with nonimmune cells. It has been demonstrated that GAS can enter epithelial cells (24, 30, 34, 38, 42, 54), so we investigated whether or not SpyA affects bacterial entry into epithelial monolayers *in vitro*. HeLa cells were infected with wild-type or *spyA* mutant bacteria at a multiplicity of infection (MOI) of ~10:1, and association and internalization into these cells was measured. After 3 h of incubation, wild-type and *spyA* mutant bacteria associated with HeLa cells in equivalent numbers (2.71×10^5 wild-type or 2.73×10^5 *spyA* mutant bacteria/well; $P = 0.95$). After treatment with antibiotics at levels that kill extracellular but not intracellular bacteria, the number of intracellular bacteria was determined. The *spyA* mutation resulted in an approximately 2-fold increase in bacterial internalization (Fig. 5). The overall rate of internalization was low but was consistent with previously published reports for M1 serotype strains (30). As with

PMNs, cytotoxicity was monitored by assessing the amount of lactate dehydrogenase released from infected cells. No difference was detected in the cytotoxicities of the two strains during the time span covered by the internalization experiments.

DISCUSSION

ADP-ribosyltransferases are important toxins for a number of Gram-positive and Gram-negative bacteria. It was previously shown that *Streptococcus pyogenes* encodes an ADP-ribosyltransferase, SpyA, which modifies a number of eukaryotic targets, including actin and vimentin (10). Here we demonstrate that subcutaneous infection of Crl:SKH1-Hr^{hr} mice with bacteria lacking *spyA* results in smaller lesions than infection with wild-type bacteria. Lesions formed after infection with *spyA* mutant bacteria began to diminish in size 1 day earlier than those generated after infection with wild-type bacteria, and the difference in size established between the two groups at these early time points was sustained until day 11. This suggests that the impact of SpyA is most pronounced during the initial interaction with the host immune system. Experiments examining mRNA levels of the chemokines CXCL1 and CCL2 and of the intermediate filament vimentin support this interpretation. At 24 h postinfection, the levels of *Cxcl1*, *Ccl2*, and *Vim* transcripts in tissue infected with mutant bacteria were elevated compared to levels in tissue infected with wild-type bacteria. By 72 h, no significant differences were detected between mRNA levels in wild-type and *spyA* mutant-infected tissues. Though the magnitude of the differences we detected was modest, similar degrees of change have been shown to alter host responses in other models. For example, mice infected with *Bordetella pertussis* lacking the pertussis toxin, another ADP-ribosyltransferase, had approximately 3 times the amount of *Cxcl1* transcript in their lungs at 6 h postinfection than mice infected with wild-type *B. pertussis*; this corresponded with an approximately 2-fold difference in neutrophil recruitment to the lungs at days 1 and 2 postinfection (2). In addition, knockdown experiments using silencing RNA directed toward vimentin showed that a 65% reduction in transcription resulted in reduced proliferation, differentiation, and phagocytic function of BM2 monoblasts (5).

CXCL1 and CCL2 are neutrophil and monocyte chemoattractants, respectively (45, 67); however, we did not detect differences in the numbers of infiltrating leukocytes. Our data do not allow us to rule out the possibility that microscopic examination of tissue is not sufficiently sensitive to detect subtle differences in numbers of leukocytes or that possible differences in the numbers of leukocytes present in the initial hours of the infectious process contributed to differences in lesion size. However, our data do suggest the possibility that functions of CXCL1 and CCL2 in addition to those on leukocyte recruitment may be involved in generating the phenotype seen during soft tissue infection. Mice deficient in CCL2 or CXCR2, a CXCL1 receptor, exhibit slower wound closure than their wild-type counterparts (13, 36). In $CCL2^{-/-}$ mice, monocyte infiltration was unchanged, but wounds demonstrated both decreased angiogenesis and delayed synthesis of collagen components (36). Furthermore, CCL2 has been shown to induce MMP-2 in dermal fibroblasts (66). In this context, our data are consistent with a pathogenic role for SpyA in delaying wound repair by reducing epithelial cell migration. A similar role has been described for C3bot, C3stau, and *Clostridium difficile* toxin A (1). Though we did not detect differences in the levels of *Mmp2* and *Fgf2* between wild type and mutant groups at 24 h, we did demonstrate lower *Mmp2* transcript levels at 72 h postinfection. This apparent inconsistency between *Mmp2* transcript levels and smaller wound size may reflect the earlier onset of lesion resolution and subsequent diminished requirement for MMP2 in the mutant-infected animals.

Vimentin is a multifunctional protein, and modulation of the expression of this protein may increase GAS virulence through a number of pathways. Vimentin-deficient mice have slower wound healing than wild-type animals (15). The protein is preferentially expressed in migrating cells in a mammary epithelial model of wound healing (19) and is upregulated and secreted in response to muscle cell injury (25). Transcription of vimentin is also induced during differentiation of the U937 and BM2 cell lines into macrophage-like cells (5, 26, 52). Activated macrophages secrete vimentin, and treatment of activated macrophages with an antivimentin antibody and treatment of maturing monoblasts with vimentin-specific small interfering RNA (siRNA) both lower production of reactive oxygen species, potent antimicrobial molecules (5, 44). Our data do not allow us to distinguish between the cell migration and immune functions of vimentin in the animal model we employed. Reduced cell migration as a result of lower *Vim* transcription is consistent with the increase in median lesion size from day 1 to day 2 seen in mice infected with wild-type bacteria. Reduced *Vim* transcription also suggests fewer monocytes undergoing maturation, consistent with the demonstrated reduction in *Ccl2* transcript levels at 24 h. It is interesting that not only does SpyA modify vimentin directly (10), but we show here that it is also associated with reduced mRNA levels *in vivo*. The mechanism by which SpyA decreases vimentin mRNA remains to be elucidated. One possibility is that SpyA-mediated modification of vimentin may alter cell signaling, since it causes the collapse of the vimentin network (L. Coye, personal communication). The vimentin network has been suggested to serve as a scaffold for signaling molecules. For example, RhoA-binding kinase α (ROKα) associates with and phosphorylates vimentin, resulting in a collapse of vimentin filaments and translocation of ROKα to the cell periphery (56), demonstrating that vimentin can serve as a framework to localize signaling molecules within the cell. Changes in the vimentin scaffold as a result of ADP ribosylation may inhibit signal transduction intended to elicit upregulation of genes important for the innate immune response.

Enhanced internalization of SpyA mutant bacteria by epithelial cells may be related to increased cytokine induction by the mutant strain. Li et al. demonstrated that intracellular *Staphylococcus aureus* induced CXCL1 expression in human epithelial cells (35). We demonstrated a SpyA-mediated reduction in the number of intracellular GAS in HeLa cells. Although invasion of epithelial cells is usually assumed to represent increased virulence, the role of intracellular streptococci during infection remains enigmatic. A number of reports demonstrate that GAS can be found intracellularly within the tonsils of patients with recurrent tonsillitis (48, 50) and that bacteria can resurge from host cells (38, 47). However, other studies have demonstrated that bacteria isolated from minor

infections are more efficient at entering host cells than those isolated from invasive infections (42), that reduced expression of capsule, streptolysin O, or NADase increases the number of intracellular bacteria but reduces cytotoxicity and/or virulence (4, 7, 28, 54), and that internalization prevents passage of bacteria through disrupted tight junctions into deeper tissue (12). Our data suggest that a reduction in intracellular bacteria *in vitro* may be associated with the decrease in chemokine production seen during *in vivo* infection with wild-type GAS and consequently increased virulence. Reduction of chemokine levels appears to be an important pathogenic mechanism for invasive GAS. The bacterium encodes a protease, SpyCEP (PrtS, ScpC), capable of cleaving a number of human CXC chemokines in addition to murine CXCL1 and CXCL2; isogenic mutants lacking SpyCEP are less virulent than their wild-type counterparts (16, 18, 27, 33). Continued work is needed to ascertain the specific role, if any, that SpyA-mediated avoidance of uptake plays in epithelial cell chemokine production.

Our data demonstrate that while SpyA is not an essential virulence factor in the Crl:SKH1-Hr^{hr} mouse subcutaneous infection model, the toxin contributes to lesion formation and thus represents another weapon in the multifaceted arsenal of *S. pyogenes.* Elucidation of the specific mechanisms by which SpyA impacts host responses will require increased understanding of SpyA secretion and possible translocation, identification of relevant modification targets, and clarification of the functional effects of ADP ribosylation on those substrates.

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