IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Genome-Wide Screens Identify Group A Streptococcus Surface Proteins Promoting Female Genital Tract Colonization and Virulence

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Group A streptococcus (GAS) is a major pathogen that impacts health and economic affairs worldwide. Although the oropharynx is the primary site of infection, GAS can colonize the female genital tract and cause severe diseases, such as puerperal sepsis, neonatal infections, and necrotizing myometritis. Our understanding of how GAS genes contribute to interaction with the primate female genital tract is limited by the lack of relevant animal models. Using two genome-wide transposon mutagenesis screens, we identified 69 GAS genes required for colonization of the primate vaginal mucosa *in vivo* and 96 genes required for infection of the uterine wall *ex vivo*. We discovered a common set of 39 genes important for GAS fitness in both environments. They include genes encoding transporters, surface proteins, transcriptional regulators, and metabolic pathways. Notably, the genes that encode the surface-exclusion protein (SpyAD) and the immunogenic secreted protein 2 (Isp2) were found to be crucial for GAS fitness in the female primate genital tract. Targeted gene deletion confirmed that isogenic mutant strains Δ spyAD and Δ isp2 are significantly impaired in ability to colonize the primate genital tract and cause uterine wall pathologic findings. Our studies identified novel GAS genes that contribute to female reproductive tract interaction that warrant translational research investigation. (*Am J Pathol* 2020; 190: 862–873; <https://doi.org/10.1016/j.ajpath.2019.12.003>)

Group A streptococcus (GAS or *Streptococcus pyogenes*) causes >700 million human infections worldwide and 15 million cases annually in the United States.^{1–3} Despite a century of research, no licensed vaccine is available. Although the oropharynx is the primary site of GAS infection, other mucosal surfaces can be colonized, sometimes leading to severe human infections and death.^{2,4,5} One example is GAS colonization of and proliferation in the female genital tract, a process that may result in puerperal sepsis (sometimes referred to as childbed fever) and/or neonatal infections.^{6–16} Although rare, puerperal sepsis and neonatal infections have high morbidity and mortality rates.^{9,11,14–16} The molecular mechanisms used by GAS to

colonize the vaginal mucosal surface and to cause invasive infections in the uterine wall are poorly understood and thus somewhat mysterious and very murky.^{4,5} Given the rarity of these cases, studies designed to discover causal mechanisms in humans have been limited. However, recent work in nonhuman primates (NHPs) has found that new GAS

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pathogenesis insight can be obtained using new relevant infection models.¹⁷

Transposon mutagenesis screens, such as conducted with TraDIS and Tnseq, are powerful tools to identify pathogen genes involved in the interaction with the host.^{18–29} Several genome-wide transposon mutagenesis screens have been successfully performed in GAS, resulting in the identification of novel genes important for growth in human blood and saliva *ex vivo* and for mouse invasive infections *in vivo*.^{23,30,31} We recently performed a systematic screen for GAS genes that contribute to necrotizing myositis in NHPs and identified many novel pathogen fitness genes with potential vaccine or therapeutic implications.¹⁷ Genome-wide study of the GAS genes that contribute to fitness in the female genital tract has not been undertaken but is warranted given the dearth of knowledge about pathogen-host interaction in this anatomic site.

One important major challenge for studying bacterial infections in the human female genital tract is the lack of a suitable experimental infection model. Recently, mouse models of vaginal colonization have been developed to study group B streptococcus and GAS genital infections.^{32–34} However, mouse infection models are unlikely to fully recapitulate primate genital tract interactions. In addition, an insufficient number of colony-forming units (CFUs) can be recovered from the mouse genital tract to generate statistically significant fitness gene data. In the current study, NHP models were developed for the study of upper and lower female genital tract interactions. These NHP female genital tract models are relevant experimental systems because GAS is a human-specific pathogen, many GAS virulence factors are specific for human and NHP targets,^{35–38} and the genital tract of NHPs is anatomically and microbiologically very similar to humans.³⁹

GAS strains have been categorized historically into serotypes based on amino-terminal sequence and serologic variation in the M protein, a surface-exposed virulence factor.⁴⁰ Epidemiologic studies conducted over decades in many geographic locations have found that in contrast to the extensive variety of M protein serotypes that cause GAS pharyngitis and other infections, the spectrum of M serotypes that cause female genital tract infection is relatively narrow. Importantly, serotype M28 GAS strains are significantly overrepresented among cases of puerperal sepsis and neonatal GAS infections.^{9,10,41,42} The molecular mechanisms underpinning the enrichment of serotype M28 strains in genital infections are very poorly understood.

We report the first use of transposon mutagenesis screens to identify GAS genes that contribute to fitness in the female NHP genital tract. We developed two new NHP models of GAS pathogen-host interactions. Using a very dense transposon mutant library generated in a genetically representative serotype M28 GAS strain that commonly causes severe human infections, we discovered novel GAS genes required for colonization of the vaginal mucosa and contributing to disease pathogenesis of the uterine wall. It is reasonable to

speculate that our findings have translational research implications.

Materials and Methods

Bacterial Strains and Growth Conditions

Serotype M28 GAS strain MGAS27961 is genetically representative of a clone that is prevalent in the United States and elsewhere.^{17,43} Strain MGAS27961 has wild-type (WT) alleles of all major transcriptional regulatory genes known to affect virulence, such as *covR*, *covS*, *ropB*, *mga*, and *rocA*. This strain was used to generate isogenic gene-deletion mutant strains. GAS strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY).

Preparation of Transposon Mutant Library Stock Cultures for NHP Infection

A total of 100 μ L of the stock M28 GAS transposon mutant library was inoculated into 500 mL of THY supplemented with 0.5 μ g/mL of erythromycin and cultured at 37°C for 8 hours. The expanded transposon mutant library was pelleted by centrifugation, washed three times with phosphate-buffered saline (PBS), and then suspended in 10 mL of PBS supplemented with 20% glycerol. The suspended mutant library was divided into 10 equivalent 1 mL aliquots, transferred to cryogenic tubes, and stored at –80°C until NHP inoculation.

NHP Vaginal Colonization Model

A new NHP model of vaginal colonization was developed. For the transposon mutant library screens, six nulliparous, female cynomolgus macaques (2 to 3 years old and 2 to 4 kg) were used. Before inoculation, NHPs received one dose of medroxyprogesterone acetate to halt ovarian function. Animals were sedated with ketamine, 10¹⁰ CFU of the M28 input library was inoculated into the vaginal vault with a sterile transfer pipet, and the animal was maintained in the prone position for 5 minutes until the fluid was absorbed into the mucosa. Animals were sampled at 24 hours after infection. To analyze the output transposon mutant library, three vaginal swabs were collected by circumferentially sweeping the mucosa, transferred to 2 mL of THY supplemented with erythromycin, and plated onto Selective Streptococcus Agar (Becton, Dickinson and Company, Franklin Lakes, NJ) plates to collect the output library. Before incubation, 100 μ L was removed, serially diluted in sterile PBS, and plated to determine the number of CFUs in the output library.

Ex Vivo Uterine Wall Infection Model

To study GAS infection in the myometrium, a new *ex vivo* uterine wall infection model was developed. Nine NHP uteri were obtained at necropsy by dissecting the fallopian tubes,

Table 1 Primers and Probes Used for Generating Isogenic Gene Deletion Mutants and for TaqMan Quantitative RT-PCR

Primer	Sequence
isp deletion-1	F: 5'-GTCCGGATCCAAAATGATTTCAAGTTTCCATCGCC-3'
isp deletion-2	R: 5'-CAGCCTGCTGAGCTGTAAAGGTACTTTTATTCATATATTAATTAGTCA-3'
isp deletion-3	F: 5'-TGACTAATTAATATATGAATAAAAGTACCTTTACAGCTCAGCAGGCTG-3'
isp deletion-4	R: 5'-GTCCGGATCCCATTTGACTGTAACCTTTGTGATTTG-3'
spyAD deletion-1	F: 5'-GTCCGGATCCAGGGCGAGATGACCAATCGGATT-3'
spyAD deletion-2	R: 5'-GACAGCTGCCAACATGACGACTCCCTGCTTAACCTGGTTGGCTTCGT-3'
spyAD deletion-3	F: 5'-ACGAAGCCAAACCAAGTTAAGCAGGGAGTCGTCATGTTGGCAGCTGTC-3'
spyAD deletion-4	R: 5'-GTCCGGATCCTTGTTTGACGCTAAATATCTTAT-3'
emm28 TaqMan-1	F: 5'-CAAGCCGTCAAGGCCTAAG-3'
emm28 TaqMan-2	R: 5'-GGCTTGCCTCTGAGATTTGT-3'
emm28 TaqMan probe	5'-6FAM-CCGTGACCTTGAAGCGTCTCGTGC-TAMRA-3'
tufA TaqMan-1	F: 5'-GTCGCTTGCCTTCATCAGTT-3'
tufA TaqMan-2	R: 5'-AGTGACGAGTTGCAGTTTCG-3'
tufA TaqMan probe	5'-6FAM-CCGCGTTCGCGTTCTTCTGGAGC-TAMRA-3'

F, forward; R, reverse.

vagina, cervix, and attached soft tissues. Uteri were rinsed with sterile PBS and transferred to a sterile petri dish. The injection site for inoculation was marked, and 100 μ L of the transposon mutant library at a concentration of 3.5×10^{10} CFU/mL was injected into the myometrium. The infected uteri were transferred to 15-mL tubes that contained 5 mL of Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with erythromycin and incubated at 37°C for 24 hours. Tissue was collected from the injection site using a sterile 8-mm biopsy punch, transferred to a 15-mL tube that contained 1 mL of PBS, and homogenized (Omni International, Kennesaw, GA). Aliquots were plated on Selective Streptococcus Agar (Becton, Dickinson and Company) to count CFUs and prepare the output library for sequencing.

Massively Parallel Sequencing and TraDIS Data Analysis

The mutant pool genomic DNA preparation and transposon insertion site sequencing were performed according to previously described procedures.³¹ The resulting sequencing reads were analyzed with the TraDIS toolkit.⁴⁴ The *tradis_comparison.R* script was used to compare the reads mapped per gene between the input pools and the output pools.⁴⁴ The GAS genes with significantly decreased mutant frequency (log2 fold-change < -1 and *q* value < 0.1) in the output transposon mutant pools were interpreted as required for GAS fitness. Illumina sequencing reads of the M28 GAS input and output libraries were deposited in the National Center for Biotechnology Information Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>; project number PRJNA596540).

Construction and Characterization of Isogenic Deletion-Mutant Strains

Isogenic deletion-mutant strains were derived from WT parental strain MGAS27961, the strain used for constructing the serotype M28 transposon insertion mutant library.

Primers used for generating the isogenic mutant strains are listed in Table 1. Isogenic mutant strains were constructed by nonpolar deletion of the target gene using allelic exchange.⁴⁵ Whole genome sequencing of all isogenic mutant strains was performed to confirm the absence of spurious mutations.

Quantitative RT-PCR Analysis

Quantitative RT-PCR analysis of *emm28* expression was performed according to previous descriptions.^{46,47} Briefly, GAS strains were grown in quadruplicate in THY broth to midexponential phase (OD600 = 0.5). RNA was extracted from GAS culture using the RNeasy kit (Qiagen, Hilden, Germany). The purified RNA was converted to cDNA with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The sequences of the primers and probes used for quantitative PCR are listed in Table 1.

Western Immunoblot Analysis of SPN and SLO

Western immunoblot analysis of secreted toxins *Streptococcus pyogenes* NAD⁺-glycohydrolase (SPN) and streptolysin O (SLO) was performed as previously described.⁴⁸ GAS strains were grown in THY broth to midexponential phase (OD600 = 0.5). A total of 10 μ L of the culture supernatant from each strain was used for SDS-PAGE and subsequent immunoblotting.

SpeB Protease Activity Assay

SpeB protease activity was assessed according to previously described methods.⁴⁹ GAS strains grown to midexponential phase were stabbed into milk agar plates, and the protease activity of each strain was analyzed after overnight incubation at 37°C.

Vaginal Infection of NHPs with Isogenic Mutant Strains

Following a similar protocol as used for the TraDIS screen, parental WT and isogenic mutant strains were compared in the NHP model of vaginal colonization. First, 12 nulliparous, female cynomolgus macaques (2 to 3 years old and 2 to 4 kg) were used to compare the fitness of the WT and *spyAD* and *isp2* isogenic deletion-mutant strains ($n = 4$ animals per strain). All NHPs were given one dose of medroxyprogesterone acetate before inoculation. Animals were randomized to strain treatment groups, sedated with ketamine, inoculated in the vagina with 10^9 CFU of the indicated strain, and maintained in a prone position for 5 minutes until the fluid was absorbed into the mucosa. Animals were sampled on days 1, 2, 4, and 7 after inoculation. At each time point, the vaginal mucosa was visually examined by a veterinarian using a bivalve nasal cone speculum, and three vaginal swabs were collected by circumferentially sweeping the vaginal mucosa. The swabs were transferred to 1 mL of PBS, serially diluted, and plated for counting.

Infection of NHP Uteri *ex Vivo* with Isogenic Mutant Strains

WT and isogenic mutant strains were compared using the *ex vivo* uterine wall infection model described above. Nine uteri extracted from female NHPs at necropsy were used to

compare the WT and each of the two isogenic mutant strains ($n = 3$ uteri per strain). Each uterus was inoculated in the myometrium with 10^9 CFU of the indicated strain and incubated in Dulbecco's modified Eagle's medium for 24 hours. Tissue was collected from the injection site with a sterile 8-mm biopsy punch and homogenized to enumerate CFU counts. For histopathologic analysis, a full-thickness section of tissue taken from the inoculation site was fixed in 10% phosphate-buffered formalin, embedded in paraffin using standard automated instruments, and reviewed by a pathologist (R.J.O.) blinded to the strain treatment groups.

Skeletal Muscle Infection of NHPs with Isogenic Mutant Strains

The virulence of the parental WT strain and the two isogenic deletion-mutant strains was assessed in the NHP necrotizing myositis infection model. Animals were randomly assigned to different strain treatment groups and received 10^8 CFU/kg of one strain (WT or isogenic mutant) in the lower limb. Each strain was tested in triplicate. The animals were observed continuously and necropsied at 24 hours after inoculation.

Histopathologic Analysis

For histologic evaluation, lesions were excised and visually inspected. Lesions (necrotic muscle) were measured in 3 dimensions, and volume was calculated using the formula

Figure 1 TraDIS analysis of group A streptococcus (GAS) gene fitness in female nonhuman primate (NHP) genital tract. **A** and **B**: Volcano-plot summary of the changes in mutant abundance for each of the genes in the output mutant pools. Gene mutations (transposon insertions) that confer significantly decreased fitness (blue circles) during vaginal colonization (**A**) and uterine smooth muscle wall infection (**B**) are highlighted. **C** and **D**: Functional categorization of the GAS fitness genes identified *in vivo* in the NHP female genital tract (**C**) and *ex vivo* in the uterine wall (**D**).

Figure 2 Group A streptococcus (GAS) genes that contribute to primate female genital tract mucosal infection and myometritis and skeletal muscle necrotizing myositis. **A:** Venn diagram comparison of 69 genes required for primate female genital tract mucosal infection with 96 genes required for uterine wall infection. **B:** Venn diagram comparison of GAS genes required for female genital tract infection with 116 genes required for necrotizing myositis. Representative fitness genes assigned to each category are shown. Genes selected for further validation and investigations are highlighted in red.

for an ellipsoid. Tissue taken from the inoculation site was fixed in 10% phosphate-buffered formalin and embedded in paraffin using standard automated instruments as described previously.¹⁷ To obtain the quantitative CFU data, diseased muscle obtained from the inoculation site was weighed and homogenized (Omni International) in 1 mL of PBS. CFUs were determined by plating serial dilutions of the homogenate. Statistical differences between strain groups were determined using the *U*-test.

Statistical Analysis

Results of the CFU recovery from the NHP vaginal mucosa are expressed as means $\pm$ SEM, with statistically significant differences determined using repeated-measures analysis of variance (Prism 6; GraphPad Software, San Diego, CA). Results of CFU recovery from the infected uteri or muscle are expressed as means $\pm$ SEM, with statistically significant differences determined using the *U*-test (Prism 6). $P < 0.05$ was considered statistically significant.

Study Approvals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Houston Methodist Research Institute (protocol AUP-1217-0058).

Results

Genome-Wide Transposon Mutagenesis Screen Identifies GAS Genes Required for Vaginal Colonization and Uterine Wall Infection

We previously generated a highly saturated transposon insertion mutant library in MGAS27961, a serotype M28 strain that is genetically representative of a phylogenetic lineage that causes abundant human infections.⁴³ To understand the molecular basis of GAS interaction with the vaginal mucosa, the M28 transposon mutant library in the female primate genital tract was screened to identify genes that contribute to GAS fitness in this host anatomic niche. Six female cynomolgus macaques were infected in the genital tract with the transposon mutant library. Swabs were obtained from the vagina of each animal 24 hours after inoculation. GAS cells recovered from the vaginal swabs constituted the output mutant pools. TraDIS was used to analyze the change in the composition of the output mutant pools relative to the input mutant pools. Transposon-inactivated genes associated with significantly decreased fitness (transposon frequency log2 fold change < -1 and q value < 0.1) in the output mutant pools were interpreted as contributing to genital tract colonization. The TraDIS analysis identified 69 genes that contribute to GAS fitness in the vaginal mucosal surface of NHPs (Figures 1 and 2, Supplemental Table S1).

Necrotizing myometritis is a devastating disease with very high morbidity and mortality rates. As a first step toward gaining information about GAS genes that contribute to this disease, an *ex vivo* uterine wall infection model was developed using explanted NHP uteri. Using this infection model, an analogous TraDIS screen was performed to identify GAS genes required for infection pathogenesis in the uterine wall *ex vivo*. Eight uteri recovered from NHPs at necropsy were infected in the uterine wall with the M28 GAS transposon mutant library. After 24 hours of incubation at 37°C, GAS cells recovered from each infected uterus were collected as the output mutant pools. TraDIS analysis identified 96 genes that contribute to GAS fitness during uterine wall infection *ex vivo* (Figures 1 and 2 and Supplemental Table S2).

Functional categorization of the genes required for vaginal colonization and those required for uterine wall infection found that they encode various proven or putative transporters, transcriptional regulators, sensor kinase proteins, metabolic enzymes (nucleotide metabolism, amino acid metabolism, and carbohydrate metabolism), iron-sulfur cluster assembling proteins, and bacterial surface-displayed proteins (Figure 1).

Comparison of GAS Genes Required for Vaginal Colonization, Uterine Wall Infection, and Skeletal Muscle Necrotizing Myositis

Comparing the 69 vaginal tract fitness genes with the 96 uterine wall fitness genes found that a common set (ie, overlap) of 39 genes is required in both conditions (Figure 2A). This group includes genes that encode substrate transporters, metabolic enzymes, gene regulators, Fe-S cluster assembly proteins, and GAS secreted surface-exposed protein (SpyAD) and immunogenic secreted protein 2 (Isp2). We recently identified 116 serotype M28 GAS genes required for bacterial fitness during NHP skeletal muscle necrotizing myositis.¹⁷ Comparing the fitness genes required for female genital tract infection (vaginal colonization and uterine wall infection) with the 116 necrotizing myositis genes found that most genital tract fitness genes did not overlap with genes required for necrotizing myositis (Figure 2B). Many genes were uniquely important in the female genital tract environments, including encoding sensor kinase SptS, transcriptional regulator ArgR2, and iron-sulfur cluster assembly proteins (Figure 2B). Conversely, 21 genes are required for both genital tract infection and necrotizing myositis, including genes encoding metabolic enzymes (*glgP*, *purB*, and *glnA*), transcriptional regulators (*adcR*, *perR*, and *purR*), and bacterial surface-exposed proteins (*spyAD* and *isp2*) (Figure 2B). Seventy-eight GAS genes required for necrotizing myositis were found to be likely dispensable for female genital tract colonization, including genes for a variety of amino acid transporters (*Spy0014*, *metQNP*, *braB*, and *ssiT*) and a manganese transporter (*mtsABC*) (Figure 2B).

Contribution of SpyAD and Isp2 to GAS Fitness in the NHP Genital Tract Environments

Bacterial surface-exposed proteins are attractive therapeutic and vaccine targets. Our TraDIS screen results suggest genes encoding SpyAD and Isp2, two GAS surface proteins,^{50–52} contribute to the GAS fitness in the female genital tract. To definitively determine whether SpyAD and Isp2 participate in GAS colonization of the primate female genital tract, isogenic gene deletion mutant strains Δ *spyAD* and Δ *isp2* were generated in parental strain MGAS27961 (Figure 3A). The two isogenic mutants do not have a growth defect in rich medium THY (Figure 3B). Compared with the WT parental strain, the *spyAD* and *isp2* deletion mutants do not have a statistically significant change in the transcript level of *emm28*, the gene encoding antiphagocytic M protein⁵³ (Figure 3C). In addition, the two isogenic mutants do not have a marked change in the production of secreted virulence factors SPN, SLO, and cysteine protease SpeB (Figure 3, D and E). Four female NHPs were infected each in the genital tract with parental strain MGAS27961, the isogenic *spyAD* or *isp2* deletion-mutant strain. Compared with the parental strain, both isogenic mutant strains were significantly impaired in capacity to colonize the NHP vaginal mucosa (Figure 4A).

To validate the role of the *spyAD* and *isp2* genes to GAS fitness in the uterine wall infection model, the growth of each of the *spyAD* and *isp2* isogenic mutant strains in the myometrium of explanted NHP uteri was examined. Consistent with the TraDIS screen results, the isogenic mutants Δ *spyAD* and Δ *isp2* were significantly attenuated for growth in the uterine wall (Figure 4B). The WT strain caused large foci of bacterial growth associated with necrotic myometrium, whereas the isogenic mutants Δ *spyAD* and Δ *isp2* caused a markedly smaller area of myometrium destruction (Figure 4C). Taken together, these data confirm that *spyAD* and *isp2* contribute significantly to GAS fitness in these two NHP genital tract environments.

SpyAD and Isp2 Contribute Significantly to GAS Necrotizing Myositis

In addition to their contribution to genital tract infections, the results of our previous genome-wide screens suggest that *spyAD* and *isp2* also contribute to GAS fitness during necrotizing myositis.¹⁷ To determine whether SpyAD and Isp2 also play a role in necrotizing myositis, the virulence of the parental WT strain and isogenic gene-deletion mutant strains was assessed using a NHP necrotizing myositis infection model.⁵⁴ Consistent with the initial genome-wide screen result,¹⁷ isogenic mutant strains Δ *spyAD* and Δ *isp2* were significantly attenuated in causing necrotizing myositis in NHPs. Compared with the WT parental strain, significantly fewer CFUs of each isogenic mutant strain were recovered from the inoculation site (Figure 5A). In addition, compared with the WT parental strain, each of the two

Figure 3 *In vitro* phenotypes of the isogenic gene deletion mutants. **A:** Schematic showing the gene neighborhood of *spyAD* and *isp2* genes and the deleted regions of isogenic mutants $\Delta spyAD$ and $\Delta isp2$. **B:** The isogenic gene-deletion mutant strains have no significant growth defect in rich medium Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). **C:** Isogenic mutant strains $\Delta spyAD$ and $\Delta isp2$ have no significant change in *emm28* transcript level. **D** and **E:** The two isogenic mutant strains do not have a marked change in the production of secreted toxins *Streptococcus pyogenes* NAD⁺-glycohydrolase (SPN), streptolysin O (SLO) (shown by Western immunoblotting), and cysteine protease SpeB (shown by SpeB protease activity assay). *n* = 4 (one-way analysis of variance). WT, wild type.

mutant strains caused significantly smaller lesions characterized by less tissue destruction (Figure 5, B and C).

Genes Encoding SpyAD and Isp2 Are Ubiquitous in GAS Isolates

The distribution of the *spyAD* and *isp2* genes was examined in i) 200 closed GAS genomes that represented 77 *emm* types in the National Center for Biotechnology Information microbial genome database (www.ncbi.nlm.nih.gov/genome/microbes) as of August 1, 2019, ii) the CDC's Active Bacterial Core Surveillance invasive GAS isolates that covered 61 *emm* types from 2015,⁵⁵ and iii) our intercontinental collection of 2,095 serotype M28 GAS strains.⁴³ Using the Basic Local Alignment Search Tool (BLAST), it was found that *spyAD* and *isp2* are present in 100% of the GAS strains of the three populations mentioned above (Supplemental Table S3).

Discussion

Little is known about GAS molecular pathogenesis in the female lower and upper genital tract. Mouse models of infection have been used relatively recently to study GAS–female genital tract interactions. For example, mouse infection models found that IL-17A contributes to the control of GAS vaginal colonization.⁵⁶ Leukotriene B4 plays an

important role in GAS puerperal sepsis.⁵⁷ A transcriptome study using a mouse model identified a GAS surface protein that contributed to vaginal colonization.⁵⁸ However, because of phylogenetic distance and anatomic and immunologic differences, it is unclear whether mouse models of infection adequately recapitulate GAS infections in humans. To address this knowledge deficit, we developed two new NHP infection models and performed genome-wide screens that successfully identified GAS genes that contribute to colonization of the NHP vaginal tract and infection in the uterine wall.

A significant portion of the identified GAS fitness genes that contribute to female NHP genital tract colonization and uterine wall infection encode proven or putative transporters (Figure 1), including transporters for metal ions (*adcB*, *adcC*), and oligopeptides (*oppABCD*) (Figure 2). We recently found that GAS transporters also play a pivotal role during experimental NHP skeletal muscle necrotizing myositis.¹⁷ Collectively, these results suggest that nutrient acquisition from the host is fundamental for GAS to colonize and establish infections in these anatomic sites. GAS transporters that contribute to colonization and infection warrant further study for potential vaccine or therapeutic use.

GAS can colonize and infect multiple distinct human anatomic sites. It is reasonable to speculate that the spectrum of genes required for GAS to grow on the vaginal

Figure 4 Validation of the contribution of *spyAD* and *isp2* to group A streptococcus (GAS) fitness in the nonhuman primate (NHP) genital tract. **A:** The ability of wild-type (WT) parental strain and isogenic mutant strains $\Delta spyAD$ and $\Delta isp2$ to colonize NHP vaginal epithelial mucosa *in vivo*. **B:** Growth of the WT parental and isogenic mutant strains in the NHP uterine wall *ex vivo*. **C:** Micrographs of uterine wall lesions caused by the parental WT strain and isogenic gene-deletion mutant strains. The WT strain is associated with larger foci of necrotic myometrium with higher numbers of organisms in the NHP uterine wall *ex vivo* (circled areas). Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 4$ (**A**); $n = 3$ (**B**). $*P < 0.05$ versus WT (Kruskal-Wallis test or *U*-test). Original magnification: $\times 4$ (**C**, top row); $\times 40$ (**C**, bottom row). H&E, hematoxylin and eosin.

mucosa, in the uterine wall, and in the skeletal muscle might be different. To identify potential niche-specific genes critical for GAS fitness in the female genital tract environments, the GAS genes required for NHP vaginal tract colonization and uterine wall infection were first compared (Figure 2A).

The results indicate that 39 (57%) of the same genes required for vaginal mucosal colonization are also important for uterine wall infection, suggesting some similarity between the vaginal mucosa and myometrium environments. Conversely, the comparison also identified genes that are

Figure 5 Validation of the contribution of *spyAD* and *isp2* to group A streptococcus (GAS) virulence during necrotizing myositis. **A:** Colony-forming units (CFUs) recovered from the necrotizing myositis lesions caused by wild-type (WT) parental and isogenic mutant strains in nonhuman primate (NHP). **B:** Volume of necrotizing myositis lesions caused by WT and each isogenic gene-deletion mutant strain. **C:** Micrographs of necrotizing myositis lesions caused by the parental WT strain and isogenic gene-deletion mutant strains (hematoxylin and eosin staining). The WT strain causes larger lesions with more tissue destruction (boxed areas). Data are expressed as means $\pm$ SEM (**A** and **B**). $n = 4$ (**A** and **B**). $*P < 0.05$ versus WT (*U*-test). Original magnification, $\times 4$.

uniquely important for vaginal colonization or for causing uterine wall pathologic findings. For example, genes for zinc acquisition (*adcB*, *adcC*, and *htpA*) are important for vaginal colonization, whereas the gene that encode zinc efflux (*czcD*) is required for GAS fitness in the myometrium, suggesting that zinc availability differs between the vaginal mucosa and myometrium. Interestingly, a recent study using mouse models also found that GAS zinc efflux and zinc import are important in different anatomic niches.⁵⁹ GAS genes required for the two genital tract environments were next compared with those that contribute to NHP skeletal muscle necrotizing myositis (Figure 2B). The results indicate that the two-component regulatory system sensor kinase SptS,⁶⁰ transcriptional regulator ArgR2, and Fe-S cluster assembly proteins are required for GAS genital tract infections but not for skeletal muscle fitness (Figure 2B). Conversely, amino acid transporters (*Spy0014*, *metQNP*, *braB*, and *ssiT*) are critical for GAS survival during skeletal muscle necrotizing myositis but less so for GAS genital tract infections (Figure 2B). Further studies using isogenic gene deletion mutants are necessary to validate these observations. The comparison also identified a set of 21 GAS genes that are required in each of the three infection models (Figure 2B). This set of genes warrants further study as translational research targets.

It was discovered that two GAS surface-displayed proteins, Isp2 and SpyAD, contribute significantly to GAS colonization of the NHP female genital tract. The two genes are members of the 21 genes that contribute to fitness in the three NHP infection models. Use of isogenic mutant strains documented that Isp2 and SpyAD are critical for GAS to cause necrotizing myositis in NHPs (Figure 4).¹⁷ Proteomic analysis found that Isp2 is one of the most abundant secreted extracellular proteins made by GAS.⁶¹ Isp2 has a CHAP domain,⁶² which is often present in enzymes involved in peptidoglycan hydrolysis, suggesting that Isp2 plays a role in bacterial cell wall modification, but this has not been proven. SpyAD is a GAS cell wall protein that has been investigated as a vaccine candidate.^{63,64} Vaccination of mice with purified recombinant SpyAD confers significant protection against intranasal infection with multiple GAS serotypes.^{63,65} SpyAD has been proposed to function as an adhesin.⁵¹ However, the precise mechanism of SpyAD and Isp2 interaction with the female genital tract is unclear but is under investigation. Analysis of publicly available GAS genomes, the CDC's collection of invasive GAS strains, and our own collection of strains found that genes that encode SpyAD and Isp2 are ubiquitously present in GAS isolates (Supplemental Table S3). Consistent with our observation, a previous study found that the *spyAD* gene is present in >99% of isolates in a large global GAS collection.⁶⁶ The high gene presence rate, surface localization, and roles in virulence make SpyAD and Isp2 attractive vaccine and therapeutic targets.

Serotype M28 GAS strains have a 37.4-Kb integrative-conjugative element designated as region of difference 2 (RD2).^{41,67} Close homologs of several surface proteins that

encode genes in RD2 (such as the GAS surface protein and the R28 protein) are also present in many group B streptococcus strains,^{68,69} a major cause of female genital tract and neonatal infections. A recent study reported that deletion of the entire RD2 region conferred decreased ability of an M28 GAS strain to adhere *in vitro* to immortalized human vaginal epithelial cells and to colonize the female genital tract in a mouse model of infection.⁷⁰ Our screen did not identify genes in the RD2 region as contributing factors for GAS colonization in this NHP female genital tract infection model. At least three possibilities may explain this observation. First, only genes that contribute to GAS fitness during the first 24 hours of infection in the NHP vagina were screened. If genes in the RD2 region contribute to the long-term persistence of GAS in this site, this screen may not detect them. Second, the nature of our transposon mutagenesis screens is mixed infections. If a determinant that contributes to GAS fitness is a secreted protein, our screen may not identify this factor because of trans-complementation of mutant strains by products made by other strains in the inoculum that retains a WT phenotype for genes in the RD2 element. This finding may be an especially important matter in the context of RD2, an element that encodes seven secreted proteins.^{41,69} A third possibility is that cell culture and mouse models simply do not adequately recapitulate the female primate condition. Future studies using the NHP vaginal model of infection and isogenic gene deletion mutants are needed to definitively clarify the contribution of RD2 element genes to M28 GAS female genital tract infections.

To summarize, we developed two new NHP models for the study of GAS infection biology and used transposon mutant library screens to identify pathogen genes that contribute to colonization of the NHP vaginal tract mucosa and infection in the uterine wall. Using isogenic mutant strains, it was confirmed that genes encoding two bacterial surface proteins (Isp2 and SpyAD) are crucial for GAS vaginal colonization, myometrium infection, and necrotizing myositis. Our findings may lead to better ways to treat female GAS genital tract infections. We believe the NHP models of genital tract infection and transposon mutagenesis screens could be used to discover fitness and virulence determinants of other important female genital tract pathogens, such as group B streptococcus. Together, such studies could also make important contributions to our understanding of the molecular basis of puerperal sepsis, a disease that has an unusually rich history in medicine because of Semmelweis' pioneering studies.^{71,72}

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Author Contributions

L.Z. designed the studies, performed and analyzed TraDIS experiments, constructed and characterized isogenic mutant strains, and wrote the manuscript; R.J.O. planned and conducted experiments involving the NHPs, analyzed resulting data, and wrote the manuscript; M.O.S. sequenced the isogenic mutant strains and provided technical support for the NHP studies; S.L.K., P.Y., and L.P. constructed and characterized isogenic mutant strains; C.C.C. provided extensive technical support for all phases of the study; A.R.L.C. and A.S.W. provided intellectual guidance on the TraDIS data analysis; L.J. oversaw and performed the NHP experiments; J.M.M. designed the studies, analyzed experiments, wrote the manuscript, and oversaw the project; L.Z. and R.J.O. made substantial scientific contributions to this work and share the first author byline position.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.12.003>.

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