Regulation of Inhibition of Neutrophil Infiltration by the Two-Component Regulatory System CovRS in Subcutaneous Murine Infection with Group A Streptococcus

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Hypervirulent invasive group A streptococcus (GAS) isolates inhibit neutrophil infiltration more than pharyngitis isolates do, and the molecular basis of this difference is not well understood. This study was designed to first determine whether natural null mutations of the two-component regulatory system CovRS is responsible for the enhancement of the inhibition of neutrophil recruitment seen in hypervirulent GAS. Next, we examined the role of CovRS-regulated interleukin-8/CXC chemokine peptidase (SpyCEP), C5a peptidase (ScpA), and platelet-activating factor acetylhydrolase (SsE) in the enhanced innate immune evasion. Invasive isolate MGAS5005 induces less neutrophil infiltration and produced a greater lesion area than pharyngitis isolate MGAS2221 in subcutaneous infections of mice. It is known that MGAS5005, but not MGAS2221, has a natural 1-bp deletion in the covS gene. Replacement of covSΔ1bp in MGAS5005 with wild-type covS resulted in the MGAS2221 phenotype. Deletion of covS from MGAS2221 resulted in the MGAS5005 phenotype. Tests of single, double, and triple deletion mutants of the MGAS5005 sse, spyCEP, and scpA genes found that SsE plays a more important role than SpyCEP and ScpA in the inhibition of neutrophil recruitment and that SsE, SpyCEP, and ScpA do not have synergistic effects on innate immune evasion by MGAS5005. Deletion of sse, but not spyCEP or scpA, of MGAS2221 enhances neutrophil recruitment. Thus, covS null mutations can cause substantial inhibition of neutrophil recruitment by enhancing the expression of the chemottractant-degrading virulence factors, and SsE, but not SpyCEP or ScpA, is required for CovRS-regulated GAS inhibition of neutrophil infiltration.

Group A streptococcus (GAS) commonly causes relatively mild pharyngitis and superficial skin infections. This major human pathogen also causes approximately 10,000 cases of severe invasive infections, such as necrotizing fasciitis, sepsis, and toxic shock syndrome, annually in the United States (1). Necrotizing fasciitis is a rapidly progressive infection of the skin, subcutaneous and deep soft tissue, and muscle and leads to systemic dissemination (2). Innate immune invasion by hypervirulent GAS plays a critical role in severe invasive infections. Neutrophil infiltrate is sparse in streptococcal necrotizing fasciitis (3–5). This severe inhibition of neutrophil recruitment can be modeled in experimental animal infections (3, 6, 7) but not pharyngitis isolates (7). Peptidases ScpA and SpyCEP (also known as ScpC) produced by GAS degrade the chemotactic C5a peptide and interleukin-8 (IL-8)/CXC chemokines, respectively, and are believed to contribute to inhibition of neutrophil recruitment (3, 8–11). The secreted esterase SsE of GAS, a protective antigen (12), targets platelet-activating factor to critically contribute to GAS inhibition of neutrophil recruitment and skin invasion (7, 13). GAS also resists phagocytosis by neutrophils through the hyaluronic acid capsule and surface M protein (14, 15), kills neutrophils through streptolysins S and O (16, 17), and escapes neutrophil extracellular traps through DNases (18). Despite these advances, the molecular basis of innate immune evasion by hypervirulent GAS isolates is not fully understood. Furthermore, it is not known whether SpyCEP and ScpA also critically contribute to the inhibition of neutrophil infiltration by hypervirulent GAS isolates and if SpyCEP, ScpA, and SsE synergistically contribute to the inhibition of neutrophil recruitment in severe invasive infections.

Strains isolated from invasive infections have a high frequency of mutations in the two-component regulatory system CovRS (also known as CsrRS) (19, 20), and covRS mutations also readily arise during experimental animal infections (21, 22). Clinical isolates with a covRS mutation or deletion are usually hypervirulent. CovRS negatively regulates many virulence factors, including the capsule synthase HasA, streptolysin S, protease SpeB, DNase Sda1, IgG proteinase Mac, SpyCEP, ScpA, and SsE (13, 18, 22–26). Some covR mutations enhance virulence by relieving the CovR repression of virulence factor genes (27, 28). In contrast to the effects of CovR mutations on the expression of virulence genes, covS null mutations both up- and downregulate distinct subsets of CovR-repressed genes (26). Loss of SpeB production and enhancement of the production of the hyaluronic acid capsule and SsE, as results of covRS mutations/deletions, are critical factors in the progress of invasive GAS infections (13, 27, 28). Whether SpyCEP and ScpA are required for virulence and skin invasion of hypervirulent GAS isolates is not known.

We hypothesize that covS null mutation/deletion-enhanced expression of SpyCEP, ScpA, and SsE critically contributes to the...
enhanced innate immune evasion and virulence of GAS strains isolated from severe invasive infections. To test this hypothesis, we first performed a reciprocal analysis of the effect of covS deletion on neutrophil infiltration, virulence, and skin invasion by using two representative strains of a M1T1 subclone, MGAS5005, with a natural 1-bp deletion in covS, and MGAS2221, with the wild-type (WT) covS gene (22). We then examined the relative and synergetic contributions of SpyCEP, ScpA, and Sse to MGAS5005 inhibition of neutrophil infiltration, virulence, and skin invasion. We found that the 1-bp deletion in covS of MGAS5005 is the cause of the that strain's enhanced innate immune evasion, skin invasion, and virulence. Sse, but not SpyCEP or ScpA, is critical for the phenotype of MGAS5005. These results provide information about the basis of GAS innate immune evasion and the progression of invasive GAS infections.

**MATERIALS AND METHODS**

**Bacterial strains and growth.** MGAS5005 and MGAS2221 are representative isolates of a prevalent M1T1 subclone from an invasive-infection case in Ontario and a scarlet fever patient in Australia, respectively (26). The sse gene deletion mutants of MGAS5005 (MGAS5005sH9004) and MGAS2221 (MGAS2221sH9004) have been described previously (7, 13). MGAS5005sH9004 has also been described previously (26). These strains and their derivatives were grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37°C in 5% CO₂. Tryptose agar with 5% sheep blood and THY agar were used as solid media.

**Generation of spyCEP, scpA, and sse deletion mutants.** Upstream and downstream flanking fragments of an internal 301-bp fragment of the spyCEP gene (bases 100 to 400) to be deleted were amplified by PCR by using MGAS5005 genomic DNA and primer pairs 5'-TTTCTGAGCTGCTTGAATTTCCGAAATCAGGGCAAAATTGACATTCCAG-3' and 5'-GTTAACGCTTCGTTGTCATCGTCTG-3', respectively. The deletion of covS of MGAS5005 is the cause of the that strain's enhanced innate immune evasion, skin invasion, and virulence. Sse, but not SpyCEP or ScpA, is critical for the phenotype of MGAS5005. These results provide information about the basis of GAS innate immune evasion and the progression of invasive GAS infections.

**Mouse infections.** All animal experimental procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (31). The protocol for the animal procedures was approved by the Institutional Animal Care and Use Committee at Montana State University (permit 2011-57).

GAS bacteria grown to mid-exponential phase in THY were harvested by centrifugation, washed three times with pyrogen-free Dulbecco's phosphate-buffered saline (DPBS), and resuspended in DPBS in the desired doses. Because outbred CD-1 Swiss mice were cheaper than BALB/c mice while MGAS5005 shows similar inhibition of neutrophil recruitment in both CD-1 and BALB/c mice, we used outbred, 5-week-old CD-1 female mice from Charles River Laboratories in this study. Groups of 5 or 10 mice were subcutaneously infected with 0.2 ml of GAS suspension with an optical density at 600 nm (OD₆₀₀) of 0.9 or about 10⁷ CFU of MGAS5005 and/or their isogenic mutant in 0.2 ml DPBS with an OD₆₀₀ slightly higher or lower than 0.9, depending on the mutant. Actual inocula were determined by plating. For virulence comparisons, infected mice were monitored daily for 14 days to determine survival rates. For other analyses, mice infected were euthanized at 24 h after inoculation to collect skin, liver, and spleen samples. Liver and spleen samples were homogenized in DPBS by using a Kontes pestle. GAS bacterial numbers in the homogenized samples were determined by plating. To measure lesion sizes and neutrophil recruitment, the skin around the infection site was peeled off and the whole infection area was recognized by the boundary of the inflammation and excised; the area was traced on paper for measurement of the infection area by weighing the traced paper.

**Quantification of neutrophil infiltration.** Numbers of recruited neutrophils in the excised skin were estimated by the myeloperoxidase assay as described previously (7, 32). Skin samples were ground in 0.5% hexadecyltrimethylammonium bromide in 50 mM potassium and sonicated on ice for 15 s to extract myeloperoxidase. The samples were frozen and thawed three times, sonicated, and centrifuged at 16,000 × g for 5 min, resulting in the supernatant for the following myeloperoxidase assay. The supernatant was added to 0.2 ml of 50 mM phosphate buffer, pH 6.0, containing the extracted myeloperoxidase, 0.167 mg/ml o-dianisidine dihydrochloride, and 0.001% hydrogen peroxide, and the change in absorption at 460 nm (ΔA₄₆₀) with time was recorded with a SPECTRAMAX 384 Plus spectrophotometer (Molecular Devices). The myeloperoxidase activity, ΔA₄₆₀/min, was converted into the number of neutrophils by using a standard curve of myeloperoxidase activities versus known numbers of murine neutrophils isolated from bone marrow as previously described (33).

**Histological analyses.** Skin infection sites were excised with a wide margin after the skin was peeled off and fixed in 10% neutral buffered formalin for 24 h. The samples were dehydrated with ethanol, cleared with xylene, and infiltrated with paraffin using a Tissue Embedding Console System (Sakura Finetek, Inc.). The paraffin blocks were processed to obtain 4-μm sections, which were stained with hematoxylin and eosin (H&E) or with a tissue Grain stain kit from Richard-Allan Scientific according to the
should be restored if cosWT is successfully knocked in. Indeed, MGAS5005WTcosWT, like MGAS2221, produced detectable SpeB activity (Fig. 2A). The transcription of spyCEP, sse, and hasA in MGAS5005WTcosWT was 40-, 50-, and 133-fold lower than that in MGAS5005, respectively, and was similar to that in MGAS2221 (Fig. 2B), further confirming the replacement of cosΔ1bp with cosWT. Thus, we successfully replaced cosΔ1bp of MGAS5005 with the WT cosWT gene.

Next, we compared MGAS5005 and MGAS5005WTcosWT for virulence, skin invasion, and neutrophil recruitment in subcutaneous infections of mice. Most of the mice infected with MGAS5005 died, whereas all of the mice infected with MGAS5005WTcosWT survived (P = 0.012) (Fig. 2C). At 1 day after inoculation, the area of MGAS5005WTcosWT infection sites (76.5 ± 35 mm²) was 90% smaller than that of MGAS5005 infection sites (786 ± 976 mm²) (P < 0.0001) but was similar to that of MGAS2221 infection sites (85.5 ± 13.9 mm²) (P = 0.63) (Fig. 2F). In addition, the GAS load in the spleens of mice infected with MGAS5005WTcosWT was 4.6
orders of magnitude lower than that in the spleens of MGAS5005-infected mice but was similar to that in the spleens of MGAS2221 infected mice (Fig. 2E). The level of neutrophils at MGAS2221ΔcovS sites ([4.6 ± 2.5] × 10⁵ neutrophils/mm²) was 54-fold lower than that at MGAS2221 sites ([2.5 ± 1.3] × 10⁵ neutrophils/mm²) (P = 0.0440) but was similar to that at MGAS5005 infection sites ([4.0 ± 0.4] × 10⁵ neutrophils/mm²) (P = 0.4000) (Fig. 2D). The size of the MGAS2221ΔcovS infection sites (637 ± 107 mm²) was 7-fold larger than that of the MGAS2221 sites (85.5 ± 15.9 mm²) (P = 0.0006) and similar to that of MGAS5005 infection sites (786 ± 82 mm²) (P = 0.2100) (Fig. 2F). While the lesion sizes of MGAS2221 infections in Fig. 1D and 2F were similar, the lesion sizes of MGAS5005 infections in the two experiments were different. This difference was most likely due to the fluctuation of the actual inoculum size. An MGAS5005 suspension with an OD₆₀₀ of 0.9 was used in both experiments, but the number of viable MGAS5005 bacteria in the inoculum for Fig. 2F was approximately 10% higher than that in the inoculum for Fig. 1D. Despite this difference, these results clearly indicate that the phenotype of MGAS5005 in skin infection is caused by the covS null deletion and that CovRS negatively regulates the inhibition of neutrophil recruitment by GAS.

Relative contributions of SpyCEP, ScpA, and Sse to MGAS5005 skin invasion, virulence, and inhibition of neutrophil recruitment. Since the expression of spyCEP, scpA, and sse is enhanced by the covS deletion in MGAS5005, we hypothesize that SpyCEP, ScpA, and Sse contribute to the MGAS5005 phenotype. We first tested this hypothesis by determining the relative contributions of these hydrolases to MGAS5005 skin invasion, virulence, and inhibition of neutrophil recruitment. We deleted a 301-bp fragment of the spyCEP gene and a 1,240-bp fragment of the scpA gene. The mutants were identified by PCR and confirmed by DNA sequencing (data not shown). SpyCEP was deleted in MGAS5005 by Western blotting but was not found in the ΔspyCEP mutant, confirming the spyCEP deletion (data not shown). Both the spyCEP and scpA deletion mutants produced the M protein at levels that were similar to that produced by MGAS5005, as judged by Western blotting (data not shown). MGAS5005ΔspyCEP and MGAS5005ΔscpA had competitive growth indexes of 0.78 and 0.94, respectively, against MGAS5005 in a mouse air sac infection model. Thus, the deletion of spyCEP or scpA did not have a growth issue or substantially alter emm expression. The competitive growth result of the ΔspyCEP mutant confirms the previous results (10, 37).

The MGAS5005ΔspyCEP and MGAS5005ΔscpA mutants were compared in subcutaneous infections of mice with the parent strain MGAS5005 and its Δsse mutant. MGAS5005ΔspyCEP, MGAS5005ΔscpA, and MGAS5005 infection sites had similar overall pathology, showing extensive GAS spreading and inflammation, whereas the MGAS5005Δsse site was small and appeared to have robust inflammatory cell infiltrate (Fig. 3). Quantitatively, the lesion sizes of MGAS5005ΔspyCEP ([363 ± 72] mm²) and MGAS5005ΔscpA ([358 ± 78] mm²) infections were not significantly different from that of MGAS5005 infections ([331 ± 43] mm²), whereas the MGAS5005Δsse lesion size was significantly smaller ([153 ± 48] mm²) (Fig. 4C). Mice infected with MGAS5005ΔspyCEP (P = 0.9037 versus the WT) or MGAS5005ΔscpA (P = 0.9524 versus the WT) had survival curves similar to that of MGAS5005 (Fig. 4A), whereas all of the mice infected with MGAS5005Δsse survived (P < 0.0001 versus the WT). These results indicate that deletion of spyCEP and scpA did
but not SpyCEP or ScpA, is required for MGAS5005 inhibition and neutrophil distribution in MGAS5005 and MGAS5005 infections. The histological findings on MGAS5005 infection sites (2.5 × 10⁴ neutrophils/mm²) and MGAS5005 infection sites (2.6 ± 1.7 × 10⁴ neutrophils/mm²) were not different from those at MGAS5005 infection sites (2.5 ± 1.8 × 10⁴ neutrophils/mm²) (Fig. 4D). In contrast, deletion of sse significantly enhanced neutrophil ingress by 5.4-fold ([1.3 ± 0.4] × 10⁴ neutrophils/mm²).

**No alteration of the histological pattern by spyCEP deletion.** One feature of the innate immune evasion of MGAS5005 is that it can keep neutrophils at a distance. To determine whether spyCEP is required for this pattern of inhibition of neutrophil infiltration, we examined MGAS5005 and MGAS5005ΔspyCEP infection sites at 24 h after infection by H&E and Gram staining. The inoculation site of MGAS5005ΔspyCEP had a zone of scattered neutrophils at the inner side of the skin (the left side of Fig. 5A and B), which was followed by a band of amorphous material and then by a GAS-containing area (Fig. 5A and B). The bacterial territory had no neutrophils. This pattern is the same as one that was recently described at MGAS5005 infection sites (7). At the spread area, there were sparse neutrophils in both MGAS5005 (Fig. 5C and D) and MGAS5005ΔspyCEP (Fig. 5E and F) infections. This pattern of GAS and neutrophil distribution in MGAS5005 and MGAS5005ΔspyCEP is different from that in MGAS5005Δsae infection sites, where neutrophils march into the area containing bacteria (Fig. 5G and F). The histological findings on MGAS5005Δsae infection of CD-1 mice confirm our previous findings on MGAS5005Δsae infection of BALB/c mice (7). These data further indicate that Sse, but not SpyCEP, plays a critical role in innate immune evasion by MGAS5005.

**No synergistic effect of Sse, SpyCEP, and ScpA on MGAS5005 inhibition of neutrophil infiltration.** Although SpyCEP and ScpA do not individually contribute to MGAS5005 inhibition of neutrophil recruitment, they may have additive effects on innate immune evasion and thus virulence and skin invasion. To test this idea, we also generated double and triple spyCEP, scpA, and sse mutants of MGAS5005. The sse gene was deleted after the spyCEP and/or scpA genes were deleted to obtain the double and triple mutants. The ΔspyCEP ΔscpA Δsse triple mutant had a competitive index of 0.98 against the ΔspyCEP ΔscpA double mutant (data not shown), indicating that the sse deletion in the background of no spyCEP or scpA had no effect on in vivo growth. The ΔspyCEP ΔscpA Δsse double deletion mutant induced (2.8 ± 1.4) × 10⁵ neutrophils/mm² and caused lesions of (3.4 ± 0.3) mm², and these results were not significantly different from those of infections with MGAS5005, MGAS5005ΔspyCEP, and MGAS5005ΔscpA (Fig. 4D). The virulence of the ΔspyCEP ΔscpA Δsse mutant was similar to that of MGAS5005, MGAS5005ΔspyCEP, and MGAS5005ΔscpA as well (P = 0.675 versus the WT, P = 0.5161 versus the ΔspyCEP mutant, and P = 0.6478 versus the ΔscpA mutant) (Fig. 4A and B).

In contrast, the ΔspyCEP Δsse, ΔscpA Δsse, and ΔscpA Δsse mutants all induced significantly higher levels of neutrophil recruitment (Fig. 4D), caused significantly larger lesions (Fig. 4C), and had significantly attenuated virulence (Fig. 4A and B) compared with MGAS5005 and its spyCEP and scpA single and double deletion mutants. In addition, the ΔscpA Δsse mutant caused significantly larger lesions than the Δsse and ΔscpA Δsse mutants did, suggesting that the spyCEP deletion in the absence of the sse gene enhanced the invasion of skin by MGAS5005. All of these results indicate that SpyCEP and ScpA did not additively contribute to a reduction of neutrophil infiltration by Sse.

**Effects of sse, spyCEP, and scpA deletions on MGAS2221 skin invasion and inhibition of neutrophil recruitment.** The data on MGAS5005 inhibition of neutrophil recruitment suggest that CovRS regulates the inhibition of neutrophil recruitment and that Sse is an important factor in this regulation. To determine whether these findings are applicable to GAS with intact CovRS, we constructed MGAS2221ΔspyCEP and MGAS2221ΔscpA mutants and compared them with MGAS2221 and MGAS2221Δsae for skin invasion and neutrophil infiltration in subcutaneous infections of CD-1 mice. All of these mutants had normal M protein production according to Western blotting (data not shown). MGAS2221Δsae induced (6.5 ± 2.8) × 10⁴ neutrophils/mm², which was 80% higher than the (3.6 ± 1.2) × 10⁴ neutrophils/mm² at MGAS2221 infection sites (P = 0.0340), whereas the levels of neutrophils at MGAS2221ΔspyCEP ([4.0 ± 1.1] × 10⁴ neutrophils/mm²; P = 0.3068) and MGAS2221ΔscpA ([4.5 ± 1.1] × 10⁴ neutrophils/mm²; P = 0.1225) infection sites were not significantly different from those at MGAS2221 infection sites (Fig. 6B). Deletion of the sse gene slightly decreased the lesion size in CD-1 mice, but the difference was not statistically significant. Deletion of scpA had no effect on skin invasion, whereas deletion of spyCEP significantly increased lesion size (Fig. 6A), confirming previous findings (11). These data indicate that the findings on invasive GAS isolates with nonfunctional CovRS regulation are qualitatively applicable to GAS with intact CovRS. Our results also suggest that CovRS can regulate neutrophil recruitment by regulating the sse gene.

**DISCUSSION**

This report describes two findings on the innate immune evasion of GAS: (i) the natural covS null deletion in MGAS5005 confers its innate immune evasion phenotype, and (ii) Sse is a dominant factor in MGAS5005 evasion of innate immunity, and SpyCEP is required for this pattern of inhibition of neutrophil infiltration, and neutrophil distribution in MGAS5005 and MGAS5005 infections. The histological findings on MGAS5005 infection sites were not different from those at MGAS5005 infection sites (2.5 ± 1.8 × 10⁴ neutrophils/mm²) and MGAS5005 infection sites (2.6 ± 1.7 × 10⁴ neutrophils/mm²) were not different from those at MGAS5005 infection sites (2.5 ± 1.8 × 10⁴ neutrophils/mm²) (Fig. 4D). In contrast, deletion of sse significantly enhanced neutrophil ingress by 5.4-fold ([1.3 ± 0.4] × 10⁴ neutrophils/mm²).
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**FIG 4** Effects of single, double, and triple deletions of spyCEP, scpA, and sse on MGAS5005 virulence, skin invasion, and neutrophil recruitment in subcutaneous infection of mice. (A and B) Survival rates of 10 mice subcutaneously infected with $1.0 \times 10^8$ CFU MGAS5005, $1.0 \times 10^8$ CFU ΔscpA mutant bacteria, $1.5 \times 10^8$ CFU ΔspyCEP mutant bacteria, $1.4 \times 10^8$ CFU Δsse mutant bacteria, $1.6 \times 10^8$ CFU ΔspyCEP Δsse mutant bacteria, $1.6 \times 10^8$ CFU ΔscpA Δsse mutant bacteria, $1.4 \times 10^8$ CFU ΔscpA ΔspyCEP mutant bacteria, or $1.4 \times 10^8$ CFU ΔscpA ΔspyCEP Δsse mutant bacteria. (C and D) Lesion sizes (C) and neutrophil recruitment (D) at 24 h after subcutaneous infection of mice in two independent experiments. In experiment 1 (solid circles), mice were infected with $9.1 \times 10^7$ CFU MGAS5005, $1.1 \times 10^8$ CFU ΔspyCEP mutant bacteria, $9.2 \times 10^7$ CFU ΔscpA mutant bacteria, $1.7 \times 10^8$ Δsse mutant bacteria, $1.2 \times 10^8$ CFU ΔspyCEP Δsse mutant bacteria, $1.1 \times 10^8$ CFU ΔscpA Δsse mutant bacteria, $1.2 \times 10^8$ CFU ΔscpA ΔspyCEP Δsse mutant bacteria, or $1.2 \times 10^8$ CFU ΔscpA ΔspyCEP Δsse Δmutant bacteria. In experiment 2, mice were infected with $9.3 \times 10^7$ CFU MGAS5005, $1.2 \times 10^8$ CFU ΔspyCEP mutant bacteria, $1.0 \times 10^8$ CFU ΔscpA Δsse mutant bacteria, $1.5 \times 10^8$ CFU Δsse mutant bacteria, $1.1 \times 10^8$ CFU ΔscpA Δsse Δmutant bacteria, $1.0 \times 10^8$ CFU ΔscpA Δsse Δmutant bacteria, or $1.2 \times 10^8$ CFU ΔscpA Δsse Δmutant bacteria. One-way ANOVA of the PMN data: not significant, all pairs among MGAS5005 and the ΔscpA, ΔspyCEP, and ΔscpA ΔspyCEP ΔscpA mutants and pairs among the strains carrying Δsse; significant, all of the other pairs. One-way ANOVA of the lesion data: not significant, the Δsse mutant versus the ΔscpA Δsse mutant, the ΔscpA Δsse Δmutant versus the ΔscpA Δsse Δmutant, MGAS5005 versus the ΔscpA Δsse Δmutant, MGAS5005 versus the ΔscpA mutant, MGAS5005 versus the ΔscpA Δsse mutant, the ΔscpA Δsse Δmutant versus the ΔscpA Δsse Δmutant, the ΔscpA Δsse Δmutant versus the ΔscpA Δsse Δmutant; significant, all of the other pairs.

and ScpA alone and in combination do not significantly contribute to the inhibition of neutrophil infiltration of MGAS5005. In addition, the relative contributions of Sse, SpyCEP, and ScpA to the inhibition of neutrophil recruitment is correlated with their relative importance for GAS virulence and skin invasion, suggesting that reduced neutrophil ingress is a critical factor in hypervirulence and skin invasion. These findings provide insight into the molecular basis of the regulation of innate immune evasion by CovRS and innate immune evasion by hypervirulent M1T1 GAS isolates.

Sparse neutrophil infiltrate has been documented in necrotizing fasciitis patients (3–5). This phenotype of innate immune evasion can be mimicked in the mouse model of necrotizing fasciitis using invasive isolates (3, 7) but not pharyngitis isolates (7). A novel finding of this study is that covS deletion can result in the phenotype of the severe innate immune evasion, which is correlated with the severity of skin invasion and hypervirulence. This finding indicates that CovRS regulates the inhibition of neutrophil infiltration and covRS null mutations maximize the inhibition of neutrophil infiltration by releasing CovRS repression of virulence factors involved in innate immune evasion. Thus, covS null mutation-enhanced inhibition of neutrophil recruitment is an addition to the list of the determinants of CovRS mutation-mediated progression of invasive GAS infection, which include loss of SpeB production and enhanced production of the hyaluronic acid capsule and DNase Sda1 in covS null mutants (18, 27, 28).

The spyCEP and sse genes are negatively regulated by CovRS. Deletion of covS enhances the expression of spyCEP and sse by ≥40-fold (Fig. 2B), confirming the previous observations (13, 38). Even though scpA is regulated by the positive regulator Mga (39), its expression is also upregulated by covS deletion (22). Thus, the relief of the CovR repression of inhibitors of neutrophil infiltration as a result of covS null mutations is expected to be the reason for the sparse neutrophil infiltrate in hypervirulent GAS infections. We previously showed that deletion of sse enhances neutrophil recruitment and the function of Sse is partly mediated by its platelet-activating factor acetylhydrolase activity (7). Thus, it is not surprising that deletion of sse from ΔspyCEP, ΔscpA, and
FIG 5 Histological analyses showing no difference in the pattern or level of neutrophil infiltration between MGAS5005 and MGAS5005ΔspyCEP. CD1 mice were subcutaneously inoculated in the back with 1.1 × 10⁸ CFU MGAS5005, 1.0 × 10⁸ CFU MGAS5005ΔspyCEP, or 1.3 × 10⁸ CFU MGAS5005Δsse, and skin samples were collected at 24 h after inoculation. (A and B) Microscopic pictures of Gram (A)- and H&E (B)-stained MGAS5005ΔspyCEP samples at the inoculation site were each combined from three snapshots taken at a magnification of ×40. (C to F) Microscopic images of Gram (C and E)- and H&E (D and F)-stained skin samples at the spread areas of MGAS5005 (C and D) and MGAS5005ΔspyCEP (E and F) infection sites. (G and H) Microscopic images of Gram (G)- and H&E (H)-stained skin samples from an MGAS5005Δsse infection site.
However, these studies lack quantitative data on the effect of mouse model of subcutaneous infection, proposing that SpyCEP is dispensable to the inhibition of neutrophil recruitment by the hypervirulent M1T1 isolate. Our results suggest that SpyCEP is not critical for covS null mutation/deletion-induced enhancement of the inhibition of neutrophil recruitment.

Sumby et al. found that skin lesion size was increased following infection with a ΔspyCEP mutant of MGAS2221, a M1T1 isolate with the covRS genes intact (11). Our test, which used MGAS2221 and our own MGAS2221ΔspyCEP mutant, confirmed the findings of Sumby et al. in a similar dermonecrosis model, two other groups found that the lesion size was reduced with a spyCEP mutant (3, 10). The discrepancy could be due to the use of different mice and GAS isolates in these studies. Although the deletion of spyCEP in MGAS5005 did not significantly affect lesion size, the deletion of spyCEP in sse-lacking MGAS5005 significantly increased skin invasion. The impact of the spyCEP deletion on skin invasion by MGAS5005 could be masked by the high capacity of MGAS5005 to invade skin. Sumby et al. proposed that the increased lesion size in a ΔspyCEP mutant infection is caused by enhanced neutrophil infiltration as a result of spyCEP deletion (11). Our neutrophil influx data are not consistent with this proposal, suggesting that spyCEP has another functional mechanism in addition to IL-8/CXC degradation. SpyCEP has been shown to be sufficient for GAS dissemination in mouse models of muscular and intranasal infections by heterologous expression of SpyCEP in Lactococcus lactis (42). The enhanced skin invasion in the absence of SpyCEP and the persistence of SpyCEP-expressing L. lactis might be due to another function of SpyCEP in promoting GAS uptake by endothelial cells (43).

MGAS5005 grows more slowly than MGAS2221 in vitro. This growth difference is likely due to the higher consumption of energy because of the enhanced production of virulence factors by MGAS5005 as a result of the covS deletion. Although MGAS2221 grows faster, it is less virulent than MGAS5005. Thus, the ability to evade the innate immune system for in vivo survival appears to be more important for GAS virulence than the capacity to grow.

In summary, a natural covS null deletion is shown to greatly enhance the inhibition of neutrophil infiltration, skin invasion, and GAS dissemination, and Sse, but not SpyCEP or ScpA, plays a dominant role in the covS deletion-caused enhancement of GAS inhibition of neutrophil infiltration, skin invasion, and virulence. The findings indicate that CovRS regulates neutrophil infiltration and that covS deletion-enhanced expression of sse, but not the enhanced expression of spyCEP, is a critical factor in the severe inhibition of neutrophil recruitment and hypervirulence, thereby advancing our understanding of the molecular basis of innate immune evasion by GAS and the progression of invasive GAS infections.

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FIG 6 Effects of sse, spyCEP, and scpA deletions of MGAS2221 on skin lesion size (A) and neutrophil recruitment (B). The data were obtained at 24 h after the subcutaneous infection of mice with 1.5 × 10⁸ CFU MGAS2221, 1.4 × 10⁸ CFU MGAS2221ΔspyCEP, 1.4 × 10⁸ CFU MGAS2221ΔscpA, or 1.6 × 10⁸ CFU MGAS2221Δsse. ΔspyCEP ΔscpA mutants enhanced neutrophil recruitment. However, it is unexpected that SpyCEP and ScpA, both alone and in combination, did not significantly contribute to the inhibition of neutrophil recruitment and SsE is a dominant factor in MGAS5005 inhibition of neutrophil infiltration. Nonetheless, it appears to be true that the relief of CovRS repression of the sse gene as a result of the covS deletion critically contributes to the inhibition of neutrophil recruitment. Deletion of sse, but not spyCEP or scpA, of MGAS2221 significantly enhanced neutrophil recruitment, supporting the idea that the findings associated with invasive GAS isolates with nonfunctional CovRS regulation are qualitatively applicable to GAS with intact CovRS.

ScpA degrades the C5a peptide, and immunization with ScpA prevents nasopharyngeal GAS colonization of mice (40). However, scpA deletion does not affect GAS virulence in subcutaneous infection of mouse tissue (41). Therefore, the insignificant contribution of ScpA to MGAS5005 inhibition of neutrophil recruitment, virulence, and skin invasion is not surprising. However, the insignificant involvement of SpyCEP in MGAS5005 innate immune evasion is a surprise. SpyCEP degrades IL-8/CXC chemokines (3, 8, 10, 11, 36, 42). This protein reduces IL-8/CXC-induced neutrophil transmigration in vitro (10, 19) and confers resistance of GAS to killing by isolated neutrophils (10). Three studies have investigated the contribution of SpyCEP to GAS pathogenesis and inhibition of neutrophil infiltration by using the mouse model of subcutaneous infection, proposing that SpyCEP contributes to the inhibition of neutrophil recruitment (3, 10, 11). However, these studies lack quantitative data on the effect of spyCEP deletion on neutrophil ingress into GAS infection sites. Furthermore, a single spyCEP deletion mutant was not available for one of these studies (3). Thus, whether SpyCEP is a critical factor in GAS inhibition of neutrophil recruitment has not been firmly established. Our quantitative analyses of neutrophil ingress indicate that SpyCEP is dispensable to the inhibition of neutrophil recruitment by the hypervirulent M1T1 isolate. Our results suggest that SpyCEP is not critical for covS null mutation/deletion-induced enhancement of the inhibition of neutrophil recruitment.
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