Vitamin D and the Human Antimicrobial Peptide LL-37 Enhance Group A *Streptococcus* Resistance to Killing by Human Cells

John F. Love,[a,b]* **Hien J. Tran-Winkler,**[a] **and Michael R. Wessels**[a]

Division of Infectious Diseases, Boston Children's Hospital, Boston, Massachusetts, USA,[a] and Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA[b]

* Present address: John F. Love, Section of Infectious Diseases, Boston University School of Medicine, Boston Medical Center, Boston, Massachusetts, USA

ABSTRACT The CsrRS two-component regulatory system of group A *Streptococcus* (GAS; *Streptococcus pyogenes*) responds to subinhibitory concentrations of the human antimicrobial peptide LL-37. LL-37 signaling through CsrRS results in upregulation of genes that direct synthesis of virulence factors, including the hyaluronic acid capsule and streptolysin O (SLO). Here, we demonstrate that a consequence of this response is augmented GAS resistance to killing by human oropharyngeal keratinocytes, neutrophils, and macrophages. LL-37-induced upregulation of SLO and hyaluronic acid capsule significantly reduced internalization of GAS by keratinocytes and phagocytic killing by neutrophils and macrophages. Because vitamin D induces LL-37 production by macrophages, we tested its effect on macrophage killing of GAS. In contrast to the reported enhancement of macrophage function in relation to other pathogens, treatment of macrophages with $1\alpha,25$-dihydroxy-vitamin D3 paradoxically reduced the ability of macrophages to control GAS infection. These observations demonstrate that LL-37 signals through CsrRS to induce a virulence phenotype in GAS characterized by heightened resistance to ingestion and killing by both epithelial cells and phagocytes. By inducing LL-37 production in macrophages, vitamin D may contribute to this paradoxical exacerbation of GAS infection.

IMPORTANCE It remains poorly understood why group A *Streptococcus* (GAS) causes asymptomatic colonization or localized throat inflammation in most individuals but rarely progresses to invasive infection. The human antimicrobial peptide LL-37, which is produced as part of the innate immune response to GAS infection, signals through the GAS CsrRS two-component regulatory system to upregulate expression of multiple virulence factors. This study reports that two CsrRS-regulated GAS virulence factors—streptolysin O and the hyaluronic acid capsule—are critical in LL-37-induced resistance of GAS to killing by human throat epithelial cells and by neutrophils and macrophages. Vitamin D, which increases LL-37 production in macrophages, has the paradoxical effect of increasing GAS resistance to macrophage-mediated killing. In this way, the human innate immune response may promote the transition from GAS colonization to invasive infection.

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) causes a wide spectrum of clinical conditions that range from asymptomatic colonization to localized infections (such as pharyngitis and impetigo) to life-threatening invasive disease, including necrotizing fasciitis and streptococcal toxic shock syndrome (1, 2). GAS appears to be uniquely adapted to survive in the human host, as no other significant reservoir of the organism has been identified in animals or the environment. Although GAS can be isolated from other body sites under some circumstances, the oropharynx is its preferred niche and the most common site of local infection. Furthermore, isolates from invasive infections are usually identical to strains associated with pharyngitis or asymptomatic pharyngeal carriage (3, 4). The latter observation has stimulated interest in determining the bacterial and host factors that govern the transition from pharyngeal colonization or localized pharyngitis to invasive infection and systemic dissemination.

The CsrRS (or CovRS) two-component system appears to play an important role in the conversion from asymptomatic carriage or localized infection to invasive and potentially life-threatening disease. Originally identified as a regulator of hyaluronic acid capsule biosynthesis, CsrRS is now known to regulate expression of up to 15% of the GAS genome (5-8). Genes included in the CsrRS regulon vary somewhat in different GAS strains, but they typically include those encoding major virulence determinants such as streptolysin O, NAD-glycohydrolase, streptolysin S, streptokinase, SpyCEP interleukin 8 (IL-8) protease, and the hyaluronic acid capsule-biosynthetic enzymes. Isolates with spontaneous inactivating mutations in *csrR* or *csrS* have been associated with clinical invasive infections and with increased virulence in experimental infections in mice (9-11). Because these mutations result in increased expression of most CsrRS-regulated virulence factors, the regulator component CsrR is thought to act predominantly as
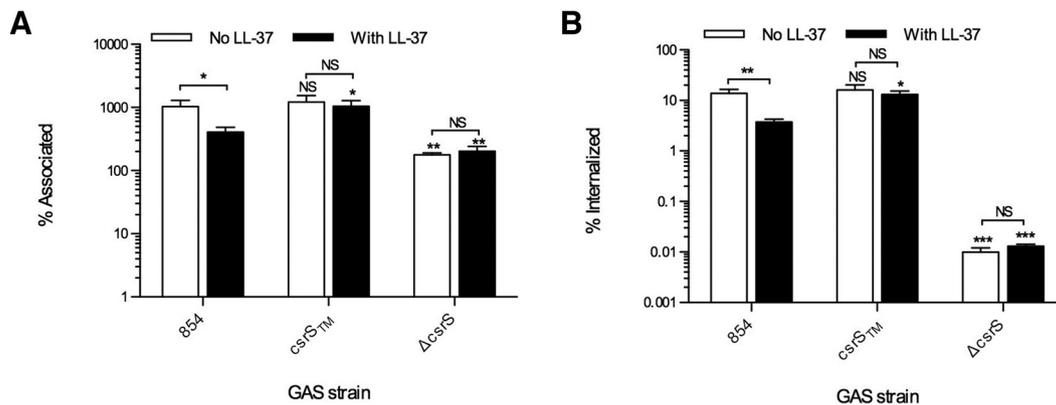
FIG 1 LL-37 decreases association and internalization of GAS (group A *Streptococcus*) by oropharyngeal keratinocytes. OKP7 cells were infected with GAS 854, 854*csrS*$_{TM}$, or 854Δ*csrS* in the absence or presence of 300 nM LL-37. Numbers of total associated CFU (A) and intracellular CFU (B) are shown as means and SEM from four independent experiments; each experiment assayed each condition in triplicate. Asterisks indicate significant differences from values for strain 854 for the same condition: *, $P < 0.05$; **, $P < 0.01$. NS, not significant.

a transcriptional repressor at regulated promoters. Exposure of GAS to high concentrations (≥ 10 mM) of extracellular magnesium results in CsrRS-dependent repression of many regulated genes, consistent with a specific signaling role for Mg^{2+} as an activating ligand for the CsrS sensor kinase (7, 12). The human cathelicidin antimicrobial peptide LL-37 functions in an opposite manner: GAS exposure to concentrations of LL-37 far below those that inhibit bacterial growth results in upregulation of the same genes that are repressed by high levels of Mg^{2+} in a CsrRS-dependent manner (13, 14).

The finding that LL-37 could stimulate a marked increase in expression of multiple virulence factors *in vitro* suggested that this innate immune effector might function *in vivo* as a specific signal to enhance GAS virulence during human infection. LL-37 has been detected in human saliva at concentrations similar to those that signal through CsrRS *in vitro* (50 to 300 nm), and local concentrations may be increased by secretion from epithelial cells or macrophages or from degranulation of neutrophils (15, 16). The effect of LL-37 signaling through CsrRS is to upregulate expression of several factors predicted to increase GAS resistance to uptake and killing by epithelial cells and professional phagocytes. The goals of this investigation were characterize the effect of LL-37 on interaction of GAS with human cell types implicated in local and systemic control of GAS proliferation and to identify critical virulence determinants in the CsrRS regulon responsible for these effects. We found that LL-37 signaling resulted in a marked increase in GAS resistance to uptake and killing by each of three key cell types—oropharyngeal keratinocytes, neutrophils, and macrophages. Thus, exposure of GAS to this innate immune effector has the paradoxical effect of enabling the organism to overcome local cellular mechanisms to control GAS proliferation. Studies of GAS isogenic mutants deficient in production of specific virulence determinants revealed that the hyaluronic acid capsule and streptolysin O (SLO) were both critical factors in LL-37-induced resistance to killing by human cells. As others have reported, we found that treatment of macrophages with vitamin D induced LL-37 production; however, in striking contrast to effects on macrophage killing of other pathogens, vitamin D treatment of macrophages promoted GAS survival.

RESULTS

LL-37 signals through CsrRS to confer GAS resistance to uptake and killing by oropharyngeal keratinocytes. During colonization or infection of the throat, GAS must adhere to the pharyngeal epithelium and resist clearance by mechanical forces such as mucous flow as well as internalization and killing by pharyngeal keratinocytes. The GAS hyaluronic acid capsule and streptolysin O have been shown to inhibit internalization of GAS by epithelial cells (17, 18). Since production of both these products is stimulated by LL-37 signaling through CsrRS, we hypothesized that exposure of GAS to LL-37 would make the bacteria more resistant to uptake and killing by oropharyngeal keratinocytes.

To test this hypothesis, we used antibiotic protection assays to assess uptake of GAS strain 854, an M1T1 clinical isolate, by human oropharyngeal keratinocytes. We found that exposure of strain 854 to 300 nM LL-37 significantly decreased both bacterial attachment and internalization by the keratinocytes (Fig. 1). We used two isogenic mutants to investigate the role of the CsrS sensor kinase in LL-37-induced resistance to internalization. Deletion of CsrS results in constitutive upregulation of several CsrRS-regulated virulence factors and lack of responsiveness to LL-37 (13, 14). Accordingly, such a mutant would be predicted to resist internalization in the presence or absence of LL-37. As expected, the *csrS* deletion mutant 854Δ*csrS* was resistant to internalization in the absence of LL-37, and resistance was not increased further by LL-37 exposure (Fig. 1B). Another mutant, strain 854*csrS*$_{TM}$, expresses a CsrS protein that has three point mutations that change a cluster of negatively charged amino acids in the extracellular domain to neutral residues, the effect of which is constitutive repression of CsrRS-regulated genes and relative refractoriness to LL-37-induced regulation of the CsrRS regulon (14). As predicted by the unresponsiveness of this mutant strain to LL-37 signaling, attachment and internalization of strain 854*csrS*$_{TM}$ were unaffected by exposure to LL-37 (Fig. 1). These results are all consistent with a model in which LL-37 signals through CsrRS to induce resistance to GAS internalization by keratinocytes.

Because both the hyaluronic acid capsule and SLO inhibit GAS internalization by oropharyngeal keratinocytes, we used isogenic mutants deficient in production of these factors to investigate
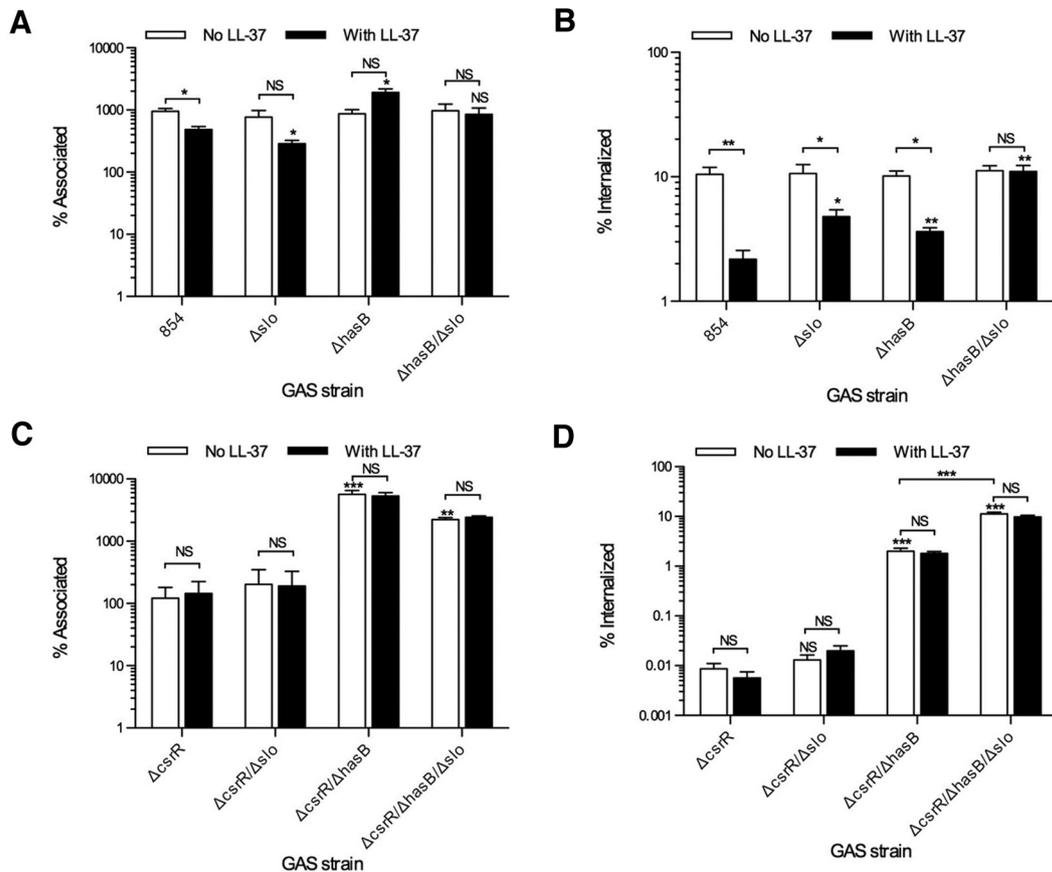
FIG 2 SLO and capsule contribute to the phenotype induced by either LL-37 exposure or inactivation of CsrR. OKP7 cells were infected with GAS in the absence or presence of 300 nM LL-37. Total associated (A) and internalized (B) CFU were measured for cells infected with GAS 854, 854Δ*slo*, 854Δ*hasB*, and 854Δ*hasB*/Δ*slo*. Similarly, total associated (C) and internalized (D) CFU were measured for cells infected with 854Δ*csrR*, 854Δ*csrR*/Δ*slo*, 854Δ*csrR*/Δ*hasB*, and 854Δ*csrR*/Δ*hasB*/Δ*slo*. Data are means and SEM from four independent experiments; each experiment assayed each condition in triplicate. Asterisks indicate significant differences from parental strain 854 or 854Δ*csrR* for the same condition: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

their contribution to the resistance to internalization induced by LL-37. Exposure of the SLO-mutant strain 854Δslo to LL-37 was less effective in inducing resistance to uptake by keratinocytes than was LL-37 treatment of wild-type strain 854 (Fig. 2B). Thus, upregulation of SLO by exposure to LL-37 contributes significantly to GAS resistance to internalization. Similarly, deletion of hyaluronic acid capsule in strain 854Δ*hasB* resulted in attenuation of LL-37-induced resistance to uptake by keratinocytes. A strain deficient in both SLO and capsule (854Δ*slo*/Δ*hasB*) was efficiently internalized in either the absence or presence of LL-37. These results indicate that SLO and capsule play critical and nonredundant roles in LL-37-induced GAS resistance to internalization by oropharyngeal keratinocytes.

Inactivation of CsrR, either by intentional deletion or spontaneous mutation, results in derepression of multiple genes in the CsrRS regulon, including the *has* operon and *slo*. This transcriptional profile is similar to that induced in wild-type GAS by exposure to LL-37 (14). Therefore, we anticipated that a CsrR deletion mutant would be resistant to internalization by keratinocytes. Indeed, strain 854Δ*csrR* was poorly associated with and highly resistant to internalization by oropharyngeal keratinocytes (Fig. 2C and D). This phenotype was not significantly altered by the deletion of *slo* (strain 854Δ*csrR*/Δ*slo*); however, deletion of *hasB*

(strain 854Δ*csrR*/Δ*hasB*) significantly increased both association and internalization. Deletion of both genes in strain 854Δ*csrR*/Δ*hasB*/Δ*slo* further increased internalization, which approached the rates seen with wild-type 854 in the absence of LL-37 exposure. Of note, the deletion of *csrR* abolished responsiveness to LL-37 exposure, consistent with the complete derepression of *slo* and the *has* operon as a result of *csrR* deletion (14).

Upregulation of hyaluronic acid capsule by LL-37 makes GAS more resistant to killing by human neutrophils. As some of the earliest inflammatory cells at the site of infection, neutrophils play a central role in controlling bacterial invasion, including that by GAS. To study the role of SLO and the hyaluronic acid capsule in resistance to neutrophil killing, isogenic mutants of strain 854 were grown in the absence or presence of LL-37 prior to exposure to human neutrophils and nonimmune serum as a complement source. In the absence of LL-37 exposure, wild-type 854, 854Δ*slo*, 854Δ*hasB*, and 854Δ*hasB*/Δ*slo* were all killed equally effectively (Fig. 3A). As demonstrated previously, wild-type strain 854 became more resistant to killing by human neutrophils when the bacteria were exposed to LL-37 (14). However, when capsule synthesis was eliminated by deletion of *hasB*, pre-exposure to LL-37 had significantly less effect, a result that supports the importance of capsule for LL-37-induced phagocytic resistance (Fig. 3A). In
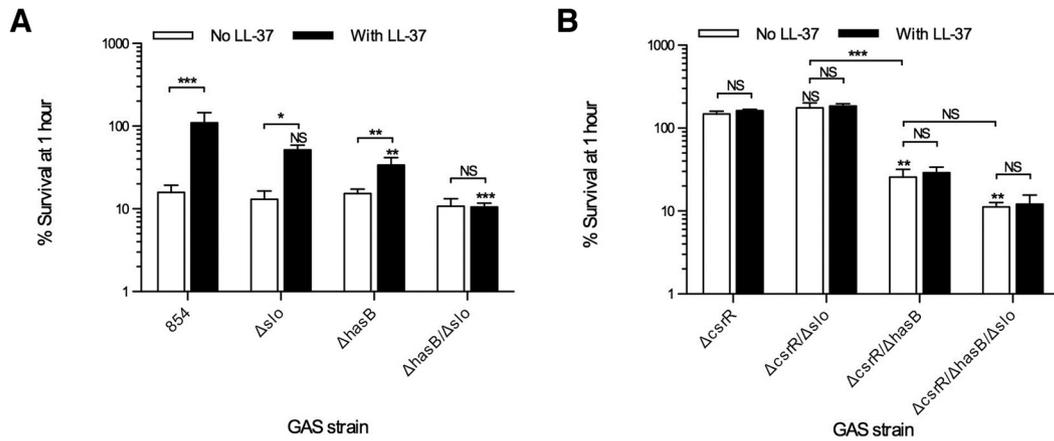
FIG 3 SLO and capsule contribute to GAS resistance to killing by neutrophils after LL-37 exposure or inactivation of *csrR*. GAS strains were grown in the absence or presence of 300 nM LL-37 prior to exposure to human neutrophils. CFU were determined by quantitative cultures at the beginning and end of a 1-h incubation and are plotted as percent survival, i.e., number of CFU at 1 h expressed as a percentage of the number present at the beginning of the experiment. (A) Infections with GAS 854, 854Δ*slo*, 854Δ*hasB*, and 854Δ*hasB*/Δ*slo*; (B) those with 854Δ*csrR*, 854Δ*csrR*/Δ*slo*, 854Δ*csrR*/Δ*hasB*, and 854Δ*csrR*/Δ*hasB*/Δ*slo*. Data are means and SEM from four independent experiments; each experiment assayed each condition in duplicate. Asterisks indicate significant differences from parental strain 854 or 854Δ*csrR* for the same condition: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

contrast, 854Δ*slo* had survival rates slightly lower than, but not statistically different from, wild-type 854. The double mutant, 854Δ*slo*/Δ*hasB*, showed no increase in resistance to killing after exposure to LL-37. Thus, capsule appears to be essential for LL-37-induced resistance to neutrophil phagocytic killing, and SLO further enhances the anti-phagocytic phenotype.

The CsrR mutant strain 854Δcsr*R* was highly resistant to neutrophil phagocytic killing. Growth of 854Δ*csrR* in LL-37 resulted in no further increase in resistance, as predicted from the complete derepression of the CsrRS regulon after deletion of *csrR* (Fig. 3B). There was no statistically significant difference between survival rates of wild-type 854 grown with LL-37 and 854Δ*csrR*. Similar to results for wild-type 854 treated with LL-37, when *hasB* was deleted from 854Δ*csrR* (Fig. 3A and B), the survival rate was significantly decreased, whereas deletion of *slo* had no significant effect (Fig. 3B). Deletion of both *slo* and *hasB* from 854Δ*csrR* created a strain (854Δ*csrR*/Δ*hasB*/Δ*slo*) that was no more resistant to neutrophil killing than wild-type 854 in the absence of LL-37 stimulation.

LL-37 induces GAS resistance to killing by macrophages. In addition to keratinocytes and neutrophils, macrophages represent some of the earliest cells GAS encounters during invasion of host tissues. SLO production by GAS is associated with cytotoxic injury to macrophages *in vitro*, and SLO has been reported to induce macrophage apoptosis, pyroptosis, and oncosis (19–21). Studies of tissue samples from patients with necrotizing fasciitis suggest that GAS can survive within tissue macrophages despite bactericidal antibiotic concentrations in the surrounding milieu (22). Macrophages have been identified as a source of LL-37 in tissue samples from healthy oropharynx and in necrotizing fasciitis caused by GAS (23, 24). Therefore, it is possible that LL-37 produced by macrophages at the site of infection signals through CsrRS to induce expression of factors that enhance GAS resistance to macrophage killing.

To evaluate the effect of LL-37 on GAS interactions with macrophages, we measured lactate dehydrogenase (LDH) release as a marker of cytotoxicity during exposure of THP-1 macrophages to

GAS in the absence or presence of exogenous LL-37. As shown in Fig. 4A, LL-37 significantly increased GAS cytotoxicity for macrophages. The CsrS triple mutant 854*csrS*$_{TM}$, in which expression of CsrRS-regulated virulence factors is repressed and which is hyporesponsive to LL-37, was not cytotoxic in the untreated condition and showed only a minimal increase in the presence of LL-37. The SLO mutant strain 854Δ*slo* also produced minimal toxicity under each condition, supporting the importance of SLO for macrophage cytotoxicity. Assays of strain 854Δ*hasB* revealed that LL-37-induced upregulation of *slo* in the absence of a concomitant increase in the antiphagocytic capsule led to a marked increase in cytotoxicity. This result suggests that the cytotoxic effect of SLO depends on phagocytic uptake of GAS, which is enhanced by elimination of the capsule. Our observations that capsule overproduction can limit SLO toxicity, as in 854Δ*csrR* above, are consistent with this model. To evaluate directly the role of GAS uptake in cytotoxicity, we blocked GAS internalization by macrophages using the actin polymerization inhibitor cytochalasin D. Blocking internalization reduced GAS-induced cytotoxicity nearly to background and abolished any increase in cytotoxicity from exposure to LL-37 (see Fig. S1 in the supplemental material). Thus, GAS-induced cytotoxicity for macrophages requires bacterial internalization, as has been reported previously (21). Consistent with this model, despite expressing approximately five times more SLO than wild-type 854, the *csrR* mutant strain 854Δ*csrR* produced only limited cytotoxicity unless capsule expression was abrogated (strain 854Δ*csrR*/Δ*hasB*) (Fig. 4C). As expected, cytotoxic injury by deletion mutants in *csrR* or *csrS* was unaffected by exposure to LL-37.

In separate experiments, we assessed the effect of LL-37 signaling on GAS survival in the presence of macrophages. THP-1 macrophages were infected with GAS in the absence or presence of exogenous LL-37 and then were treated with penicillin and gentamicin to kill extracellular bacteria. The cells were then washed and returned to antibiotic-free medium with or without LL-37. GAS that survived intracellularly and those that escaped from the cells were quantified by culturing aliquots of the cell lysate and
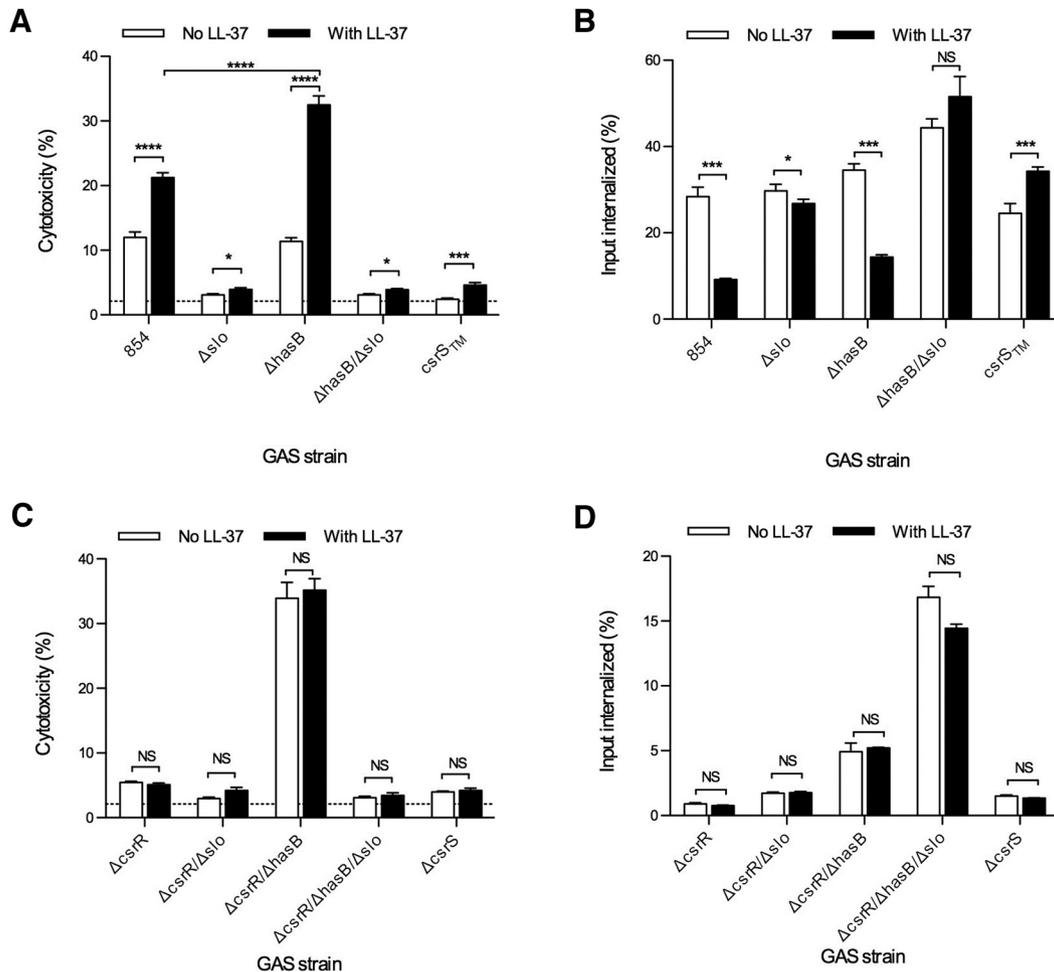
FIG 4 LL-37 increases GAS cytotoxicity for THP-1 macrophages. THP-1 cells were infected with GAS in the absence or presence of 300 nM exogenous LL-37. After infection with GAS 854, 854Δslo, 854ΔhasB, 854ΔhasB/Δslo, and 854csrS$_{TM}$, cytotoxicity was measured as LDH released into the cell culture supernatant (A), and intracellular GAS CFU were counted (B). Infections with 854ΔcsrR, 854ΔcsrR/Δslo, 854ΔcsrR/ΔhasB, 854ΔcsrR/ΔhasB/Δslo, and 854ΔcsrS were also assayed for cytotoxicity (C) and intracellular CFU (D). The background level of LDH release from cells not exposed to GAS is indicated by a broken line and did not vary between conditions. Data represent a single experiment, with five (A and C) or three (B and D) replicates, representative of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

growth medium 2 or 4 h later. As shown in Fig. 5A, wild-type strain 854 exhibited significantly better survival at 4 h in the presence of LL-37 than in its absence. In contrast, the CsrS triple mutant 854csrS$_{TM}$ survived poorly in macrophages after growth in unsupplemented medium and only slightly better in the presence of LL-37, consistent with the repressed expression of CsrRS-regulated virulence factors in this strain and its blunted response to LL-37. Deletion of slo abrogated the survival benefit conferred by LL-37 exposure, whereas a capsule-deficient strain was not significantly different from wild-type 854 with respect to survival in the absence or presence of LL-37 (Fig. 5B). Together, these results implicate SLO as the critical CsrRS-regulated determinant required for enhanced GAS survival to macrophage killing in response to LL-37. This survival benefit was absent from strains lacking a functional CsrR (ΔcsrR/ΔhasB and ΔcsrR/ΔhasB/Δslo strains), a result that supports signaling through CsrRS as the responsible mechanism and that excludes an inhibitory effect of LL-37 on the macrophages themselves (Fig. 5C).

Vitamin D treatment of macrophages augments GAS-induced cytotoxicity and GAS survival. The experiments described above utilizing exogenously added LL-37 as a signaling ligand raised the possibility that endogenous LL-37 produced by macrophages themselves might cause similar increases in macrophage cytotoxicity and enhanced GAS survival. Others have demonstrated that 1,25-dihydroxy-vitamin D3 (1,25D3) increases expression of LL-37 and its precursor hCAP18 in both human monocyte-derived macrophages and in THP-1 macrophages (25, 26). While 1,25D3-induced upregulation of macrophage cathelicidin expression has been correlated with enhanced killing of intracellular pathogens *in vitro*, its effect on killing of GAS by human macrophages is less predictable, since the enhancement of macrophage function may be counterbalanced by LL-37-induced upregulation of GAS virulence factor expression via CsrRS signaling (25, 26). To investigate the net effect of 1,25D3 on macrophage interactions with GAS, we compared GAS-induced cytotoxicity of THP-1 macrophages that had and had not been treated with 1,25D3. Upregulation of hCAP18/LL-37 was confirmed by a 38-fold (±3.1-fold) increase in gene expression by quantitative reverse transcription-PCR and by Western blot analysis of macrophage cell lysates (see Fig. S2 in the supplemental material).
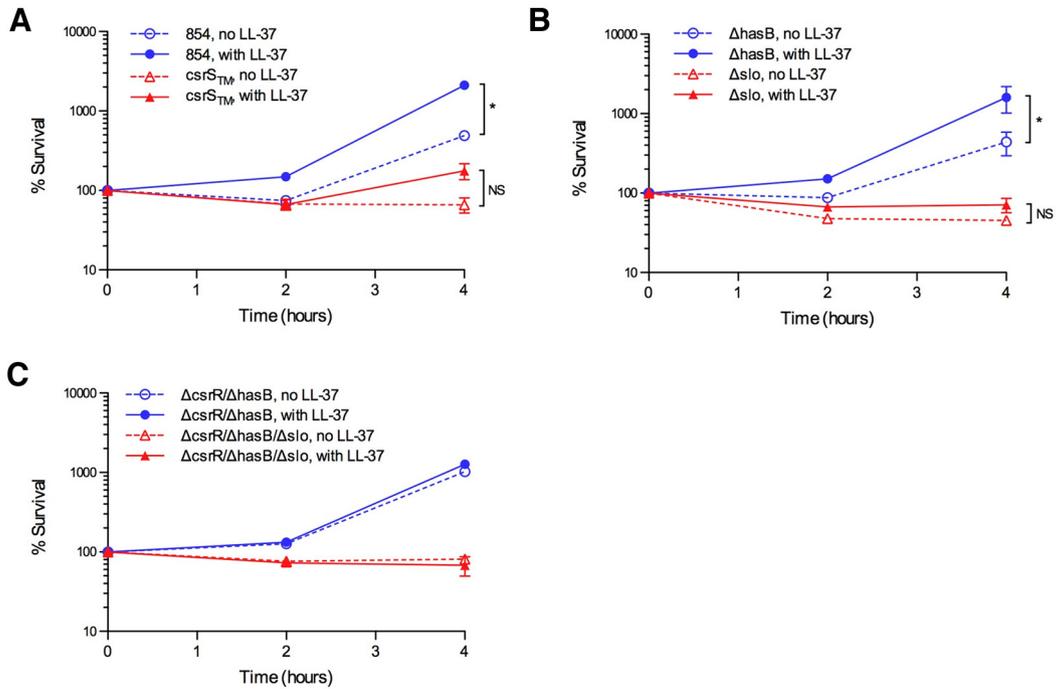
FIG 5 LL-37 makes GAS more resistant to killing by macrophages. THP-1 macrophages were infected with GAS in the absence or presence of 300 nM LL-37. GAS 854, 854csrS_TM, 854ΔhasB, 854Δslo, 854ΔcsrR, or 854ΔcsrR/ΔhasB were used for infection; data are presented in separate panels for clarity. Viable GAS released into the cell culture medium plus GAS associated with the macrophages were quantified after 90 min of incubation (time zero) and 2 and 4 h later. Data are presented for each strain as a percentage of the GAS at each time point compared to the number at time zero. Data are means and standard errors from at least three separate experiments. *, $P < 0.05$.

Macrophages that had been treated with 1,25D3 released significantly more LDH than untreated cells upon infection with GAS 854, consistent with the induction of hCAP18/LL-37 expression in the macrophages and subsequent LL-37 signaling to internalized GAS through CsrRS to increase GAS virulence gene expression (Fig. 6A; also, see Fig. S1 in the supplemental material). The SLO mutant strain 854Δslo and the CsrS triple mutant strain 854csrS_TM, which is hyporesponsive to LL-37 stimulation, induced little cytotoxicity in either untreated or 1,25D3-treated macrophages. Despite the absence of a functional CsrRS system, strain 854ΔcsrR/ΔhasB also induced increased cytotoxicity with 1,25D3-pretreated cells, although to a lesser degree than wild-type 854, a result that suggests that other vitamin D-responsive factors contribute to enhanced cytotoxicity in addition to LL-37 signaling
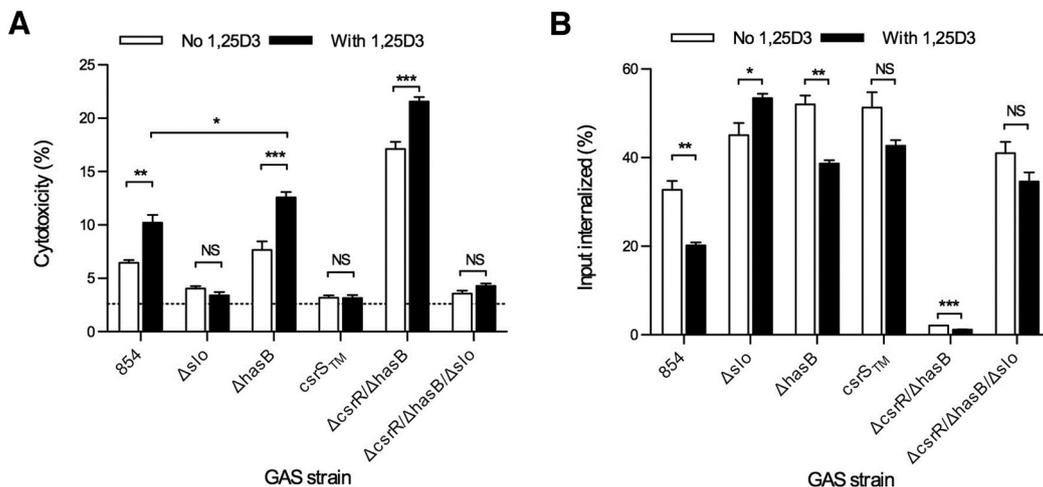
FIG 6 Pretreatment of macrophages with 1,25-dihydroxyvitamin D3 (1,25D3) increases GAS cytotoxicity. THP-1 macrophages were pretreated with 20 nM 1,25D3 for 24 h before being infected with GAS 854, 854Δslo, 854ΔhasB, 854csrS_TM, 854ΔcsrR/ΔhasB, or 854ΔcsrR/ΔhasB/Δslo. (A) Supernatants were assayed for LDH release as described above; (B) intracellular GAS were measured. GAS strain designations are as in Fig. 2. Results of a single representative experiment with five (A) or three (B) replicate measurements are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.
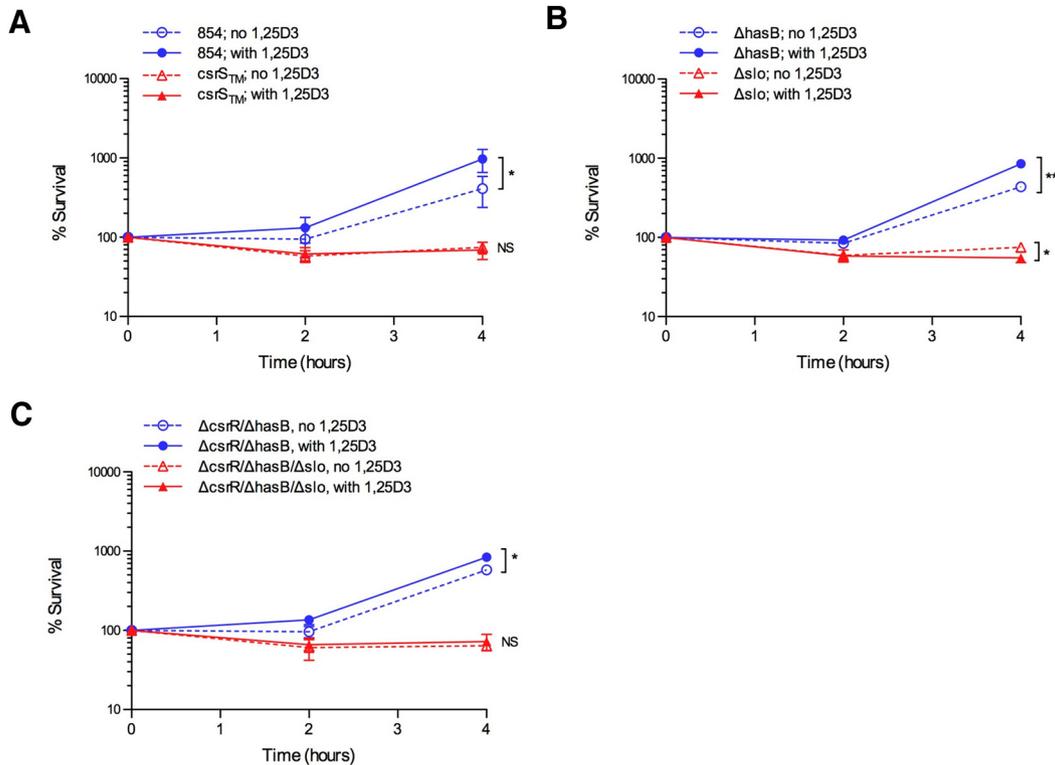
FIG 7 Pretreatment of macrophages with 1,25D3 increases GAS survival through an SLO-dependent mechanism. THP-1 macrophages were pretreated with 100 nM 1,25D3 for 24 h prior to infection with GAS. GAS 854, 854csrS$_{TM}$, 854Δ*hasB*, 854Δ*slo*, 854Δ*csrR*, and 854Δ*csrR*/Δ*hasB* were used for infection; data are presented in separate panels for clarity. Viable GAS released into the cell culture medium plus GAS associated with the macrophages were quantified after 90 min of incubation (time zero) and 2 and 4 h later. For each strain, the number of GAS at each time point, reported as a percentage of the number at time zero, is shown. Data are means and standard errors from at least three separate experiments. *, $P < 0.05$; **, $P < 0.01$. NS, not significant.

through CsrRS. The effects of 1,25D3 on GAS-induced cytotoxicity were mirrored by reciprocal effects on the efficiency of GAS internalization by infected macrophages (Fig. 6B; also, see Fig S1 in the supplemental material).

We also tested whether 1,25D3-pretreatment of macrophages affected GAS survival. As shown in Fig. 7, macrophages pretreated with 1,25D3 were significantly impaired in their ability to control proliferation of wild-type GAS 854 compared to untreated macrophages. In contrast, the *csrS* triple mutant strain 854csrS$_{TM}$, which is hypo-responsive to LL-37, was controlled effectively under either condition (Fig. 7A). The survival phenotype was dependent on expression of SLO, as the 854Δ*slo* mutant was actually killed more effectively by macrophages pretreated with 1,25D3 than by untreated macrophages (Fig. 7B). The enhanced killing of 854Δ*slo* provides evidence of increased bactericidal effector function in 1,25D3-treated macrophages, consistent with previous reports, and further highlights the paradoxical impairment in killing of wild-type GAS after 1,25D3 treatment. In contrast to SLO, capsule upregulation played little role in increased survival of GAS in macrophages, as 854Δ*hasB* behaved nearly identically to wild-type 854 (Fig. 7B). Strain 854Δ*csrR*/Δ*hasB* also survived somewhat better in 1,25D3-pretreated macrophages than in untreated cells, which suggests a role for another 1,25D3-responsive factor(s) in addition to LL-37 (Fig. 7C). Together, these experiments demonstrate that 1,25D3 treatment of human macrophages induces increased susceptibility to GAS infection, in striking contrast to the enhanced resistance to other pathogens reported previously (25, 26).

DISCUSSION

To successfully colonize and infect its human host, GAS must overcome multiple host defenses. Bacteria encounter a variety of effector molecules of the innate immune system, including antimicrobial peptides such as LL-37, on the pharyngeal mucosa. GAS has evolved a mechanism to sense LL-37 through CsrRS, a signal that is transduced into upregulation of multiple virulence genes and increased resistance to killing by various human cell types.

We found that concentrations of LL-37 comparable to those detected in human saliva significantly alter the interaction of GAS with oropharyngeal keratinocytes *in vitro*, decreasing association and internalization of GAS by these host cells. Exposure to subinhibitory concentrations of LL-37 also makes GAS more resistant to killing by neutrophils and macrophages. By decreasing internalization and killing by both epithelial cells and phagocytes, LL-37 improves GAS survival, promoting ongoing pharyngeal colonization and the potential for invasive infection.

LL-37 not only is present in saliva but also is produced by various host cells that GAS encounters within the human host. Although oropharyngeal keratinocytes do not appear to express LL-37, cutaneous keratinocytes upregulate LL-37 expression in response to injury and upon exposure to microbial products (27-29). Neutrophil β-granules are a highly concentrated source of LL-37 that can be released during phagocytosis or during neutrophil degranulation (30). Neutrophils are estimated to contain 0.6 μg of hCAP18/LL-37 per 10^6 cells, in a cellular volume of

approximately 300 μm³, yielding an approximate concentration of 100 μM (31, 32). Neutrophil extracellular traps (NETs) are lattices of extruded neutrophil chromatin studded with antimicrobial peptides, including LL-37, that represent another potential site for exposure of GAS to LL-37 *in vivo*. Concentrations of LL-37 within the neutrophil phagosome may be high enough to inhibit GAS growth and contribute to bacterial killing. However, the concentrations of LL-37 encountered by GAS in the extracellular environment or within cells that produce smaller amounts of the peptide are insufficient for effective antimicrobial activity against GAS. Rather, the subinhibitory amounts of LL-37 encountered by GAS in the latter environments are far more likely to increase virulence factor expression by signaling through CsrRS and to result in enhancement of GAS resistance to internalization and killing by host cells.

Although CsrRS regulates approximately 10 to 15% of the GAS transcriptome, we found particular importance of two virulence factors in the survival phenotype induced by LL-37: SLO and the hyaluronic acid capsule. The upregulation of *slo* reduced GAS association and internalization by oropharyngeal keratinocytes, enhanced GAS resistance to neutrophil phagocytic killing, was essential to the cytolysis of macrophages, and improved GAS survival of macrophage attack. Increased hyaluronic acid capsule production similarly decreased association and internalization by oropharyngeal keratinocytes and decreased phagocytic killing by neutrophils. LL-37 failed to increase the survival of a strain lacking both *slo* and *hasB*, so capsule and SLO appear to be essential to enhanced GAS resistance to killing by host cells. These results do not exclude potential contributions of other CsrRS-regulated virulence factors to improved GAS survival.

The transcriptional changes in GAS induced by LL-37 exposure likely have additional effects on pathogen-host interactions during infection beyond those specifically documented in this study. For example, two elements of the CsrRS regulon upregulated by LL-37 exposure, hyaluronic acid capsule and streptolysin S, have been implicated in the ability of GAS to transmigrate across epithelial layers (14, 33, 34). CsrRS signaling by LL-37 upregulates SpyCEP, which inhibits neutrophil recruitment through degradation of the chemotactic cytokine IL-8 (14, 35, 36). The *sda1* gene of M1T1 strains, which encodes a DNase active against NETs, is upregulated by LL-37 exposure, thereby contributing to GAS escape from extracellular killing by neutrophil antimicrobial peptides (14, 37, 38). In addition to the effects on keratinocytes, neutrophils, and macrophages demonstrated here, SLO inhibits maturation of dendritic cells, thereby impairing development of an adaptive immune response (39). Capsule limits binding of specific antibodies to the GAS surface protein GRAB, so upregulation of capsule by LL-37 may reduce the effectiveness of antibody-mediated opsonization (40). Taken together, these observations suggest that GAS responses to LL-37 signaling modify host-pathogen interactions at multiple stages of colonization, infection, and immune response.

Studies in mice have shown a protective role for the murine cathelicidin CRAMP in experimental GAS soft tissue infection (41, 42). However, despite its similar alpha-helical structure and antimicrobial activity, murine cathelicidin does not signal through CsrRS (13). Therefore, the mouse model does not reflect the human situation, in which the predominant effect of LL-37 is likely to be enhanced resistance to phagocytosis and increased invasiveness from upregulation of CsrRS-regulated virulence fac-

tors, rather than inhibition of GAS growth by LL-37's antimicrobial activity. Similar limitations apply to previous studies of the effect of vitamin D on resistance to GAS infection. Treatment with 1,25D3 has been shown to increase cathelicidin gene expression in human macrophages and to promote macrophage killing of *Mycobacterium tuberculosis* (25, 26). In mice, deficiency of 1,25D3 inhibited the induction of cathelicidin gene expression in skin keratinocytes and was associated with increased lesion size in experimental GAS skin infection (43). These studies support a causal relationship between vitamin D and cathelicidin expression, but the net effect on GAS virulence in human infection cannot be extrapolated from the murine model, since mouse cathelicidin lacks CsrRS signaling activity. The studies presented here demonstrate a novel instance in which effects of vitamin D on the innate immune system appear to be detrimental to the host by inducing pathogen resistance to macrophage killing.

MATERIALS AND METHODS

Ethics statement. The aspects of this study relating to human subjects were approved by the institutional review board of Boston Children's Hospital. Written informed consent was provided by study participants.

Bacterial strains and cell lines. GAS strains used in this study are listed in Table 1. GAS 854 is an M-type 1 strain originally isolated from a patient with a retroperitoneal abscess (13). GAS strains were grown at 37°C in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (THY) or on THY agar or Trypticase-soy agar (BD Biosciences) supplemented with 5% defibrinated sheep blood. *Escherichia coli* strain DH5α (Zymogen) was used for cloning and was grown in Luria broth (LB). Antibiotics were used when necessary at the following concentrations: for GAS, erythromycin at 1 μg/ml; for *E. coli*, erythromycin at 200 μg/ml and carbenicillin at 100 μg/ml.

Human cell culture. The human oropharyngeal keratinocyte cell line OKP7/bmi1/TERT (referred to here as OKP7) was described previously (44, 45). These cells were a gift of James Rheinwald and were provided through the Harvard Skin Disease Research Center. Keratinocytes were propagated in keratinocyte serum-free medium (KSFM; Invitrogen).

THP-1 cells were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen).

Chemicals and supplements. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich, dissolved in dimethyl sulfoxide (DMSO), and stored in aliquots at −20°C. 1α,25-Dihydroxyvitamin D3 (1,25D3) was purchased from Sigma-Aldrich, dissolved in ethanol, and stored as aliquots in light-impermeable tubes at −80°C. The human cathelicidin LL-37 was purchased from AnaSpec, dissolved in 0.01% acetic acid to a 1-mg/ml stock, and stored in aliquots at −20°C.

GAS mutagenesis. Deletion constructs for *hasB* and *csrS* were generated by overlap PCR using Easy-A high-fidelity DNA polymerase (Stratagene), using sets of primers listed in Table 2, as described in previous

TABLE 1 Bacterial strains used in this study

Strain	Reference	Genotype
854 (M-type 1)	13	Wild type
854*csrS*$_{TM}$	14	*csrS*(D148N/E151Q/D152N)
854Δ*csrR*	14	Δ*csrR*
854Δ*csrS*	This study	Δ*csrS*
854Δ*hasB*	This study	Δ*hasB*
854Δ*slo*	This study	Δ*slo*
854Δ*hasB*/Δ*slo*	This study	Δ*hasB* Δ*slo*
854Δ*csrR*/Δ*hasB*	This study	Δ*csrR* Δ*hasB*
854Δ*csrR*/Δ*slo*	This study	Δ*csrR* Δ*slo*
854Δ*csrR*/Δ*hasB*/Δ*slo*	This study	Δ*csrR* Δ*hasB* Δ*slo*

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence
delhasB1	GGATCCGATGTGATACTGATGTAC
delhasB2	GCATTTTCATAAAATTACTCCTTC
delhasB3	GTAATTTATGAAAATAGCAAGAGACTAGCCATGTCCTTCATC
delhasB4	GTCGACGCCCTCTTGGGTGGCTTTGAC
delcsrS1	CGAGGATCCGTTTGATTTCCTCCTGCTTGAC
delcsrS2	CTGATTTTCCATATGACTTATTTC
delcsrS3	TAAGTCATATGGAAAATCAGCAGTCTAAAGAGAGTTAGAGTAGC
delcsrS4	GAATTCGTCGACCCAATAGCAGTACTTGACACTTC

work (14). The final PCR fragments were ligated into pGEM-T (Promega) and sequenced, before being excised by digestion with SalI/BamHI and cloned into the temperature-sensitive shuttle vector pJRS233 (46). The plasmid pJSLO (47) was used to delete *slo*.

Generation of deletion mutants via allelic exchange was achieved using the recombinant shuttle plasmid pJRS233, as previously described (48). Mutant genotypes were confirmed by PCR amplification of target loci from chromosomal DNA and subsequent DNA sequencing (Genewiz). The absence of SLO in culture supernatants from Δ*slo* strains was confirmed in hemolysis assays and Western blots; NADase activity was preserved in these culture supernatants, a result that excludes a polar effect on expression of *nga*, which encodes NADase and is expressed from the same operon (see Fig. S3 in the supplemental material). The absence of hyaluronic acid capsule from Δ*hasB* strains was confirmed as described previously (18) (data not shown).

The CsrS triple mutant 854csrS$_{TM}$ and the *csrR* deletion mutant 854Δ*csrR* were described previously (14).

Opsonophagocytic killing assays with human neutrophils. GAS resistance to phagocytic killing was evaluated *in vitro* as previously described (13, 14). GAS strains were grown to early exponential phase in the absence or presence of 300 nM LL-37 in THY. At a multiplicity of infection (MOI) of 2 to 3, GAS were mixed with freshly isolated human peripheral blood leukocytes in the presence of 10% nonimmune human serum as a complement source. Aliquots were removed for quantitative culture immediately upon mixing and after 1 h of incubation at 37°C with end-over-end rotation. Results were expressed as the percentage of the inoculum surviving after 1 h. Donors of nonimmune serum were identified by the ability of their peripheral blood to support growth of GAS 854 *in vitro* (49).

Infections of oropharyngeal keratinocytes. The human oropharyngeal keratinocyte cell line OKP7 was used for antibiotic protection assays as described previously (17, 45). Cells were seeded in 24-well plates at 10^5 cells/ml and grown for 3 to 4 days to confluence at 37°C in 5% CO_2. GAS were grown to early exponential phase in THY in the absence of LL-37, pelleted, washed, resuspended in KSFM, and diluted. OKP7 cells were washed once with phosphate-buffered saline (PBS). GAS were added to cells at a MOI of 5, followed by addition of KSFM with or without 300 nM LL-37. Infections proceeded for 2 h and 15 min at 37°C in 5% CO_2 before addition of penicillin (20 μg/ml) and gentamicin (200 μg/ml) to half of the wells to kill extracellular GAS. At 3 h postinfection, cells were washed with PBS, treated with trypsin (0.25%), and lysed in water for 15 min before being serially diluted for quantitative culture on sheep blood agar. After growth for 24 h at 37°C in 5% CO_2, colonies were counted; numbers of total cell-associated GAS were determined from wells which had not received antibiotics, while numbers of internalized GAS were determined from wells treated with antibiotics.

Infection of THP-1 macrophages. THP-1 cells were seeded in 24-well plates at 5×10^5 cells/ml in RPMI with 10% FBS in the presence of 25 nM PMA. After incubation for 24 h at 37°C in 5% CO_2, cells were washed three times with PBS before the medium was replaced with RPMI with 10% FBS with or without 20 nM 1,25D3, after which the cells were incubated for an additional 24 h under identical conditions. GAS were grown in THY to early exponential phase in the absence of LL-37, pelleted,

washed, resuspended in RPMI with 10% FBS, and diluted. THP-1 macrophages were washed once with PBS before GAS were added at MOI of 1, followed by addition of RPMI with 10% FBS, with or without 300 nM LL-37. Infections proceeded for 90 min, before penicillin (20 μg/ml) and gentamicin (200 μg/ml) were added to all wells. Thirty minutes later, supernatants were removed for measurement of LDH using a cytotoxicity measurement kit (Roche). Uninfected THP-1 cells were lysed with Triton per the manufacturer's instructions, as calibration for complete cell lysis. To measure intracellular GAS, cells were washed with PBS, treated with 0.25% trypsin, and lysed in water for 15 min before being serially diluted for quantitative culture on sheep blood agar. When needed, cytochalasin D was added 30 min prior to infection at 5 μg/ml, and the medium, during infection, was supplemented to the same concentration.

For bacterial survival assays, THP-1 cells were treated and infected as described above, through antibiotic treatments. After 30 min of incubation with penicillin and gentamicin, wells were washed three times with PBS. For time zero measurements, cells were immediately treated with 0.25% trypsin and lysed in water for 15 min before being serially diluted for quantitative culture on sheep blood agar. Replicate wells had 0.5 ml of RPMI with 10% FBS (without antibiotics and with or without LL-37) added and were further incubated at 37°C for 2 or 4 h. At these time points, the culture medium was removed and set aside, while adherent well contents were trypsinized and lysed in water, as described above; the culture medium was returned to the cell lysate prior to diluting for quantitative culture.

Statistical analysis. All graphs were generated in Prism version 5.0d (GraphPad Software, Inc.), showing the mean ± standard error of the mean (SEM). The significance of the differences between groups was calculated using paired *t* tests for all experiments except LDH assays, for which nonpaired *t* tests were used.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00394-12/-/DCSupplemental.

Figure S1, TIF file, 0.8 MB.
Figure S2, TIF file, 0.7 MB.
Figure S3, TIF file, 0.8 MB.

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