IgG Protease Mac/IdeS Is Not Essential for Phagocyte Resistance or Mouse Virulence of M1T1 Group A *Streptococcus*

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ABSTRACT **The Mac/IdeS protein of group A *Streptococcus* (GAS) is a secreted cysteine protease with cleavage specificity for IgG and is highly expressed in the GAS serotype M1T1 clone, which is the serotype most frequently isolated from patients with life-threatening invasive infections. While studies of Mac/IdeS with recombinant protein have shown that the protein can potentially prevent opsonophagocytosis of GAS by neutrophils, the role of the protein in immune evasion as physiologically produced by the living organism has not been studied. Here we examined the contribution of Mac/IdeS to invasive GAS disease by generating a mutant lacking Mac/IdeS in the hyperinvasive M1T1 background. While Mac/IdeS was highly expressed and proteolytically active in the hyperinvasive strain, elimination of the bacterial protease did not significantly influence GAS phagocytic uptake, oxidative-burst induction, cathelicidin sensitivity, resistance to neutrophil or macrophage killing, or pathogenicity in pre- or postimmune mouse infectious challenges. We conclude that in the highly virulent M1T1 background, Mac/IdeS is not essential for either phagocyte resistance or virulence. Given the conservation of Mac/IdeS and homologues across GAS strains, it is possible that Mac/IdeS serves another important function in GAS ecology or contributes to virulence in other strain backgrounds.**

IMPORTANCE **Group A *Streptococcus* (GAS) causes human infections ranging from strep throat to life-threatening conditions such as flesh-eating disease and toxic shock syndrome. Common disease-associated clones of GAS can cause both mild and severe infections because of a characteristic mutation and subsequent change in the expression of several genes that develops under host immune selection. One of these genes encodes Mac/IdeS, a protease that has been shown to cleave antibodies important to the immune defense system. In this study, we found that while Mac/IdeS is highly expressed in hypervirulent GAS, it does not significantly contribute to the ability of the bacteria to survive white blood cell killing or produce invasive infection in the mouse. These data underscore the importance of correlating studies on virulence factor function with physiologic expression levels and the complexity of streptococcal pathogenesis and contribute to our overall understanding of how GAS causes disease.**

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The Gram-positive bacterium group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is responsible for a broad spectrum of diseases, ranging from the relatively mild pathologies of pharyngitis and impetigo to severe invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome (1, 2). Counting the postinfectious immunologically mediated syndrome of rheumatic heart disease, GAS is one of the top 10 causes of infectious disease morbidity and mortality, disproportionately affecting less-developed countries (1). Because preventative vaccines against GAS infections are not currently available, the immune response of the human host is a major determinant of disease outcome. The human host can develop adaptive immunity to GAS (3, 4), and anti-GAS antibodies (5), including those introduced by intravenous immunoglobulin treatment (6), can promote opsonophagocytosis and aid in the resolution of infection. Although GAS organisms in the bloodstream are particularly susceptible to antibody binding, which promotes opsonophagocytosis (7), many GAS strains with invasive potential have evolved strategies to counteract phagocyte clearance mechanisms that normally prevent bacterial dissemination (8–10).

A significant portion of the global burden of invasive GAS disease is attributable to the rise and prevalence of the M1T1 clone (11, 12). In this clone, a genetic and phenotypic switch can occur (13, 14), resulting in the strong transcriptional upregulation of genes encoding multiple virulence factors, many of which promote resistance to clearance by the innate immune system (8). These factors include the surface hyaluronic acid capsule, the pore-forming hemolysin streptolysin O, DNase Sda1, the streptococcal inhibitor of complement (SIC), and the interleukin-8 (IL-8) peptidase SpyCEP. The genetic and phenotype switch, which can be recapitulated in the mouse model of GAS infection, is attributable to mutations in the two-component transcriptional regulatory locus *covRS* (also designated *csrRS*) and is marked by strong down-regulation of a broad-spectrum protease, SpeB, that

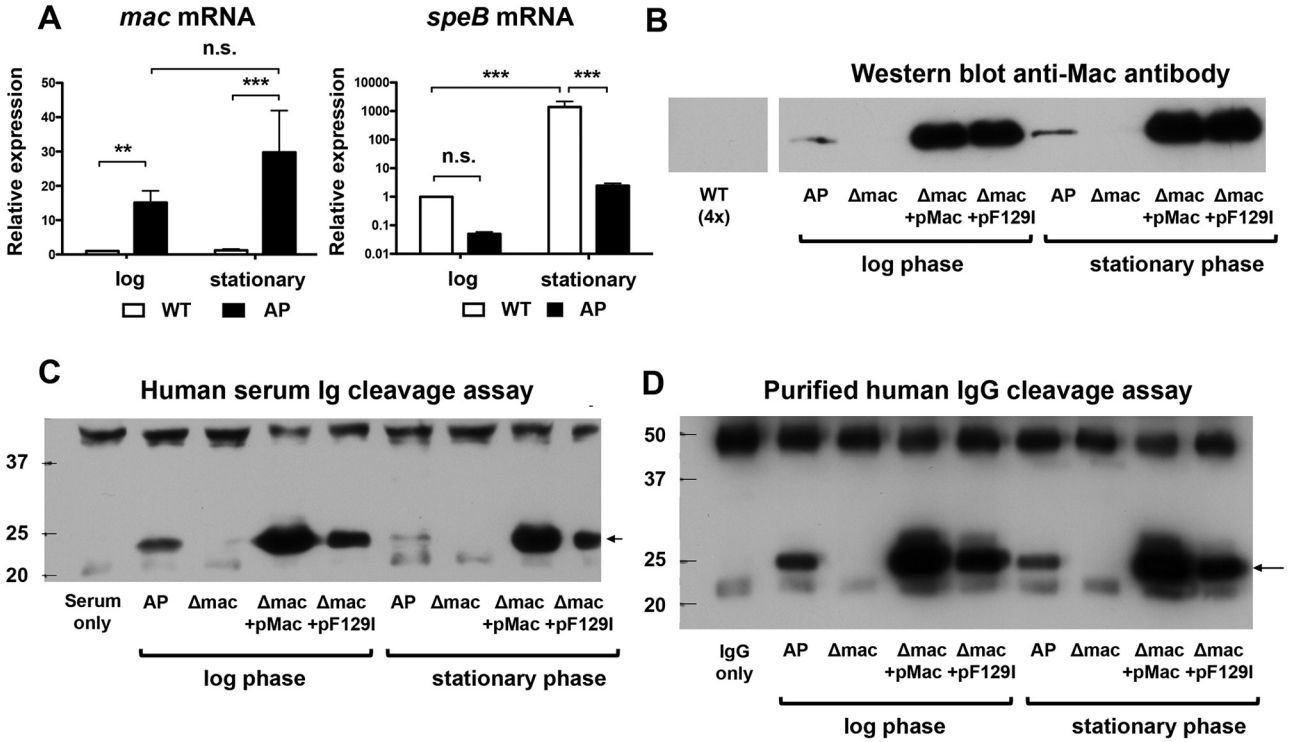

FIG 1 Mac is upregulated in the AP M1T1 GAS strain and cleaves IgG. (A) *mac* gene expression is significantly upregulated in log- and stationary-phase cultures, while *speB* is down-regulated in AP M1T1 GAS compared to the original WT strain prior to AP. Data are shown as the mean ± the standard error of the mean of three independent experiments. n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA with Tukey's multiple-comparison posttest). (B) Western blot analysis of Mac/IdeS expression in log- and stationary-phase cultures following targeted mutagenesis, AP, and complementation analysis in the M1T1 GAS background. Cell-free culture supernatants were probed with polyclonal anti-Mac antiserum. Mac/IdeS was not detected in fourfold-concentrated supernatants from the WT M1T1 GAS strain. (C and D) Cleavage of human serum Ig (C) or purified human IgG (D) by cell-free culture supernatants from log- and stationary-phase cultures of AP, Δ*mac* mutant, and complemented strains. Blots were probed with anti-human IgG antibodies. Heavy and light chains of Igs are visible, and the arrow indicates the cleavage product. In panels B to D, results of an experiment representative of at least three independent experiments are shown. The values to the left of panels C and D are molecular sizes in kilodaltons.

is otherwise capable of degrading multiple host and bacterial factors (15, 16).

One of the highly upregulated genes in the M1T1 clone associated with the *covRS* switch is that encoding the Mac protein (also designated IgG-degrading enzyme of *S. pyogenes*, IdeS) (13), a secreted 35-kDa protein with cleavage specificity for the hinge region of the IgG heavy chain (17). The name Mac derives from limited sequence homology with the α subunit of human Mac-1 (CD-11), a β2 integrin (4), and its ability to bind αvβ3 and αIIbβ3 integrins (18, 19); however, integrin binding by streptococcal Mac has so far not been implicated as a possible virulence function of the protein. Proteolytic cleavage of IgG in the hinge region by Mac/IdeS is hypothesized to prevent the recognition of antibody-opsonized bacteria by Fc receptors of immune cells and by the complement system (17). Crystallization studies of Mac/IdeS suggested that protease activity depends on dimerization (18), but recent data show that it is highly unlikely that Mac/IdeS is active as a dimer (20) or that a putative dimer would be enzymatically more active than monomeric Mac/IdeS (20, 21).

In studies with recombinant Mac/IdeS incubated with serum containing antibodies against GAS, proteolytic degradation inhibited subsequent opsonophagocytosis (4, 17, 19) and reactive oxygen species production (4, 22) by human neutrophils. However, no study examining potential virulence functions of the Mac/IdeS protein in the physiological context of expression by the native organism during the course of an infection has been reported. Our present work examined the requirement of Mac/IdeS for invasive M1T1 GAS infection through targeted mutagenesis and studies with host phagocytic cells and mouse infection models.

RESULTS

Expression and activity of Mac/IdeS. Animal passage (AP) by mouse subcutaneous infection allows the selection of M1T1 strains with *covRS* mutation, hyperencapsulation, SpeB inactivation, and virulence factor upregulation, similar to bacteria found in invasive infections (13–15). To confirm that *mac*/*ideS* transcript levels were upregulated in our prototypical M1T1 strain (5448, wild type [WT]) and its AP form, we analyzed gene expression in log- and stationary-phase cultures (Fig. 1A). We found that *mac*/*ideS* expression was significantly greater in the AP (spontaneous *covS* mutant) cultures than in the parent M1T1 strain cultures, corroborating previously published results (13). Similar high *mac*/*ideS* expression levels were present during both the log and stationary phases of AP strain growth (Fig. 1A). Conversely, cysteine protease gene *speB* expression levels were much higher in the WT strain than in the AP variant, with maximal *speB* expression in stationary-phase cultures (Fig. 1A), also in agreement with earlier data (14). To corroborate our gene expression data, we performed Western blot analyses of secreted Mac/IdeS protein expression levels in cell-free culture supernatants from WT and

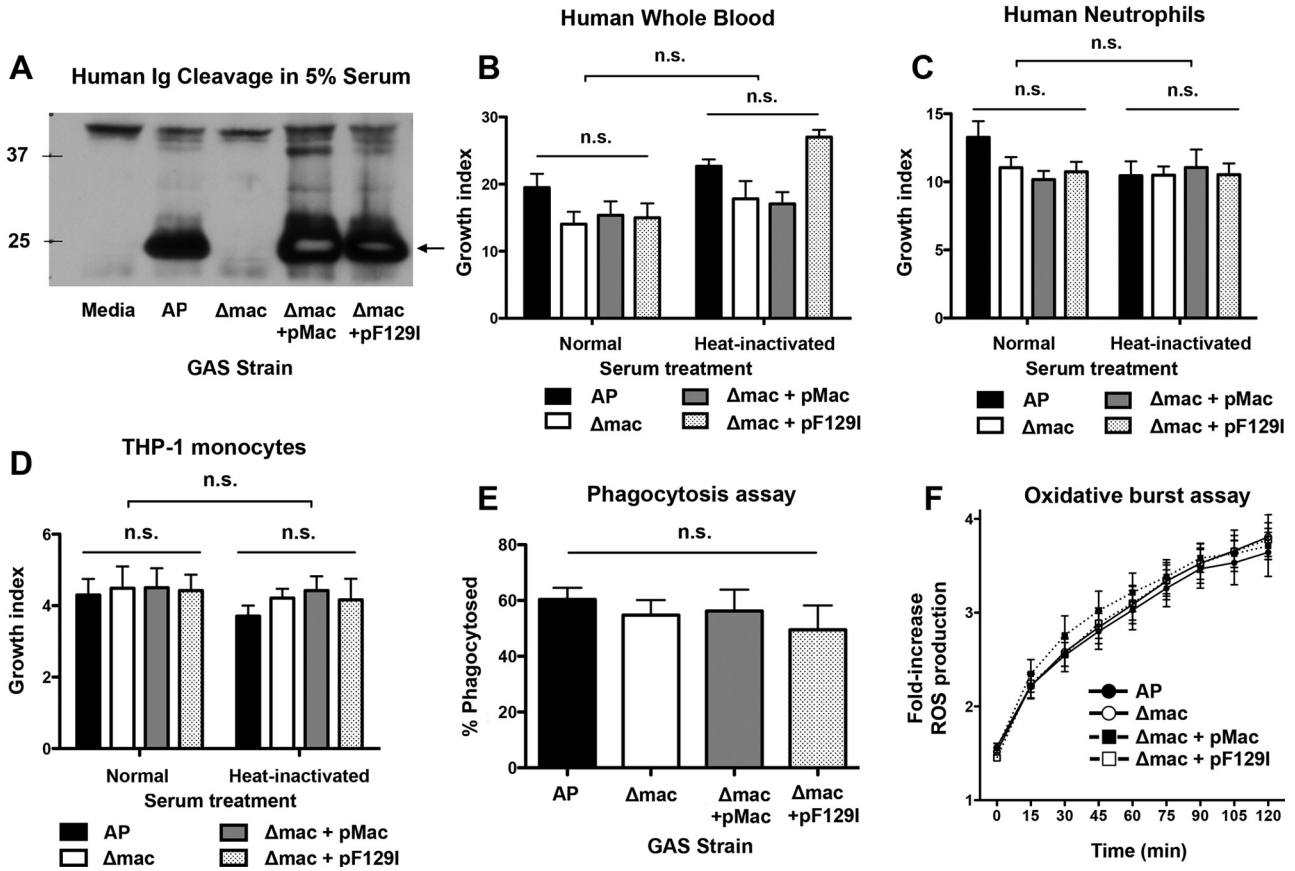

FIG 2 Mac/IdeS is not essential for M1T1 GAS resistance to phagocyte killing. (A) The AP M1T1 and complemented strains grown in 5% serum cleave serum Igs, whereas the Δ*mac* mutant strain does not. Filter-sterilized culture supernatants were probed with anti-human IgG antibodies. Heavy and light chains of Igs are visible, and the arrow indicates the cleavage product. Results of an experiment representative of at least three independent experiments are shown. (B to D) The AP, Δ*mac* mutant, and complemented strains grown in 5% serum show no differences in survival when incubated with whole blood (B), freshly isolated human neutrophils (C), or differentiated THP-1 cells (D). No differences in survival were seen whether the bacteria were grown in normal serum or heat-inactivated serum. (E and F) Phagocytosis (E) and the oxidative-burst response (F) by human neutrophils do not significantly differ upon incubation with the AP, Δ*mac* mutant, or complemented strain. In panel F, relative fluorescence is compared to that at time point 0. In panels B to F, data are shown as the mean ± the standard error of the mean of three independent experiments. Data were not significantly different between strains and serum conditions by one-way ANOVA with Tukey's multiple-comparison posttest (B to E) or by two-way ANOVA (F). n.s., not significant. The values to the left of panel A are molecular sizes in kilodaltons. ROS, reactive oxygen species.

AP GAS strains. Mac/IdeS protein was below the detection limit in WT M1T1 GAS culture supernatants, even when they were concentrated 4-fold, but was readily detected in both log- and stationary-phase culture supernatants of the AP strain (Fig. 1B). Combined, these data show that Mac/IdeS is expressed at relatively low levels by the WT M1T1 strain but that significant levels of protein are produced as the bacteria undergo the genetic switch, suggesting that Mac/IdeS could play a role in the virulence of the hyperinvasive AP form. As the expression of SpeB, which has been reported to have IgG protease activity *in vitro* (23–25), is markedly down-regulated in the AP strain (Fig. 1A), the effects of Mac/IdeS on IgG cleavage can be studied independently. Thus, we focused the remainder of our studies on the hypervirulent AP M1T1 strain.

To study the role of Mac/IdeS in a physiologic setting, precise, in-frame allelic-exchange mutagenesis was performed to eliminate the *mac* gene from GAS M1T1 strain 5448. The mutant was first constructed from the WT strain by a previously described method (26–28) and subsequently subjected to AP by murine subcutaneous infection. The SpeB-negative colonies recovered were confirmed through DNA sequencing to possess an inactivating mutation within *covS* paralleling that of the AP strain (data not shown). The resulting strain in the AP M1T1 background was designated the Δ*mac* mutant strain. For complementation analysis, *mac*/*ideS* was cloned into an expression plasmid (pMac) and transformed into the Δ*mac* mutant strain. To analyze the role of dimerization in Mac/IdeS activity, a plasmid containing a modified *mac*/*ideS* gene harboring a single point mutation (pF129I) that has been suggested to disrupt dimer formation (18) was likewise used to complement the Δ*mac* mutant strain. Mac protein was absent from the Δ*mac* mutant and appropriately expressed by both complemented strains, as shown by Western blot analysis of cell-free culture supernatants (Fig. 1B).

We next examined whether IgG cleavage in response to concentrations of Mac/IdeS such as those produced endogenously by the panel of M1T1 GAS organisms expressing or lacking Mac could be observed. Cell-free culture supernatants from the AP, Δ*mac* mutant, and complemented strains were incubated

with either pooled human serum (Fig. 1C) or purified human IgG (Fig. 1D), and cleavage products were visualized by Western blot analysis with an anti-human IgG antibody. The parent AP M1T1 GAS strain produced a cleavage product of approximately 23 to 24 kDa (Fig. 1C and D), in agreement with previously published data obtained with recombinant Mac/IdeS (17). The Δ*mac* mutant lost cleavage activity, whereas activity was restored in the complemented strains (Fig. 1C and D), including the strain expressing the putative dimerization mutant Mac protein. Overexpression of Mac/IdeS on the multicopy complementation plasmids corresponded to increased IgG cleavage activity (Fig. 1C and D). No IgG cleavage activity by fourfold-concentrated supernatant from log-phase cultures of the nonpassaged WT M1T1 strain was observed (data not shown), in agreement with the low expression levels of both Mac/IdeS (Fig. 1A and B) and SpeB (Fig. 1A). Our results show that the AP M1T1 (*covS* mutant) strains produce Mac/IdeS at sufficient concentrations to exert observable IgG cleavage activity. Our data further confirm that dimerization of Mac/IdeS is not required for cleavage activity (20).

Mac is not essential for invasive M1T1 GAS resistance to phagocyte killing. To assess the role of Mac/IdeS in the context of GAS-phagocyte interactions, the AP, Δ*mac* mutant, and complemented strains were grown to log phase in bacteriologic medium containing 5% normal human serum to provide a level of bacterial opsonization and antibody-mediated activation of the complement pathway. No significant growth differences between strains in medium containing serum were noted (data not shown). We confirmed that the human serum used in the growth medium contained significant titers of anti-GAS antibodies by enzyme-linked immunosorbent assay (ELISA) (see Table S1 in the supplemental material). Cell-free supernatant obtained from strains cultured in the 5% serum-containing medium showed that IgG cleavage activity was measurable during the growth phase (Fig. 2A).

Whole bacterial cultures (bacteria plus culture supernatant containing secreted Mac) were then added to a variety of phagocytic cell types, and bacterial survival was assessed. To assess the possible contributions of antibody-mediated complement system activation to opsonophagocytosis, control experiments with heat-inactivated serum were performed in parallel. No significant difference in survival in whole blood (Fig. 2B), isolated human neutrophils (Fig. 2C), or differentiated monocytes (THP-1 cells, Fig. 2D) was observed between any of the strains. The degree of bacterial survival in whole blood and with isolated neutrophils was donor dependent (data not shown) but GAS antibody independent, as all of the donors exhibited similar GAS antibody titers (see Table S1 in the supplemental material). There were also no significant differences in the survival of the strains grown in either normal or heat-inactivated human serum (Fig. 2B to D), indicating that the complement system does not play a role in phagocytic clearance of the bacteria in this model system and may play a limited role in an infection setting. Finally, no significant differences in survival with phagocytic cells were found between strains in the nonpassaged (intact *covRS*) background (see Fig. S1 in the supplemental material), demonstrating that Mac/IdeS also does not contribute to virulence in a WT noninvasive strain in this model system.

It has been previously reported that Mac/IdeS cleaves IgG and prevents opsonophagocytosis and a subsequent oxidative burst (4). Generation of 1/2 Fc fragments by Mac/IdeS can also prime neutrophils to produce an oxidative-burst response (22). We found that opsonization significantly enhanced the ability of phagocytic cells to control bacterial growth (see Fig. S2A in the supplemental material), suggesting that Mac could play a role in preventing opsonophagocytosis. However, opsonization did not affect the ability of the cells to phagocytose the AP strain (see Fig. S2B), and with our panel of AP, Δ*mac* mutant, and complemented strains, we demonstrated that neither phagocytosis of the bacteria by isolated human neutrophils (Fig. 2E) nor the oxidative-burst response of the neutrophils (Fig. 2F) significantly differed. Similarities in the phagocytosis of the strains support our finding that there is no significant difference in phagocyte killing (Fig. 2B to E). Finally, no difference between strains in the killing kinetics, MICs, or minimal bactericidal concentrations (MBCs) of the antimicrobial peptide LL-37 was observed (Table 1). Taken together, our results suggest that the increased expression of Mac/IdeS in the AP M1T1 GAS strain, although possessing measurable IgG cleavage activity, does not significantly contribute to bacterial survival in the presence of phagocytes.

TABLE 1 LL-37 MICs and MBCs for the strains used in this study[a]

Strain	MIC (μM)	MBC (μM)
AP	8–16	8–16
Δ*mac* mutant	8–16	8–16
Δ*mac* mutant/pMac	8–16	8–16
Δ*mac* mutant/pF129I	8–16	8–16

[a] No significant MIC or MBC differences between strains were found by one-way ANOVA. Variations in MIC and MBC reflect individual experimental variability.

Mac/IdeS is not required for M1T1 GAS virulence in a mouse model of infection. We next examined the requirement of Mac/IdeS for GAS virulence *in vivo*. Mice were subcutaneously challenged with either the AP M1T1 strain or the Δ*mac* mutant strain. Given the overexpression of multiple virulence factors, the AP M1T1 GAS strain is hyperinvasive, and the infection quickly spreads systemically, with mice succumbing within 3 to 4 days (Fig. 3A). No significant differences in the kinetics of survival between mice infected with the AP strain and those infected with the Δ*mac* mutant were noted (Fig. 3A), indicating that in a GAS-naive mouse, Mac does not obviously contribute to virulence. To test the hypothesis that anti-GAS antibodies are necessary to evaluate the full effect of Mac/IdeS (an IgG protease) on infection, mice were immunized with heat-killed GAS over a course of 4 weeks. We confirmed that the anti-GAS serum antibody titers after the immunization protocol were markedly higher than those of the preimmune serum controls (Fig. 3B). Effective GAS antibody production was confirmed by the significantly better survival of the immunized mice than the nonimmunized mice (Fig. 3A and C, $P > 0.001$), but no significant difference in survival between immunized mice infected with the AP strain and those infected with the Δ*mac* mutant strain was observed (Fig. 3C). Cleavage data obtained with purified mouse IgGs indicate that Mac/IdeS can only cleave murine IgG subtypes 2a to c and 3 (29). The inability of Mac/IdeS to cleave all mouse IgG subtypes may reduce its potential to contribute to virulence in our infection model. Nevertheless, the mouse infection data support the *in vitro* data, showing that Mac/IdeS does not significantly contribute to the invasiveness phenotype of the hypervirulent AP M1T1 GAS strain.

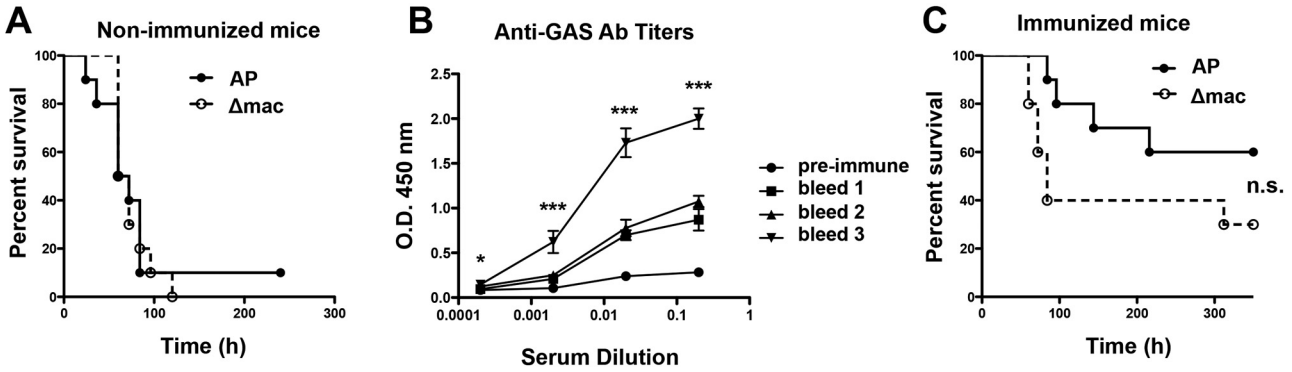

FIG 3 Mac/IdeS is not essential for GAS M1T1 virulence in a murine model of infection. (A) No difference in survival was observed in nonimmunized mice infected subcutaneously with either the AP or the Δ*mac* mutant strain. (B) Mice were immunized over the course of 4 weeks, and anti-GAS antibody (Ab) titers in immunized mice (bleed 3) were significantly higher than those in preimmune serum. (C) No difference in survival was observed in immunized mice infected subcutaneously with either the AP or the Δ*mac* mutant strain. For each panel, 10 mice were used per strain. In panel B, data are shown as the mean ± the standard error of the mean. *, $P < 0.05$; ***, $P < 0.001$ (for differences between bleed 3 and preimmune serum samples by one-way ANOVA with Tukey's multiple-comparison posttest). Panels A and C were analyzed by the log rank (Mantel-Cox) test.

DISCUSSION

In GAS serotype M1T1, the transition from localized to invasive infection can be accompanied by mutations in the genes encoding the two-component regulatory system CovRS (13–16) and transcriptional changes in about 10% of the entire bacterial genome (13, 14) and can be recapitulated by AP (13–15). These transcriptional changes include strong upregulation of genes encoding many proven virulence factors (8) and a marked upregulation of the gene encoding Mac/IdeS (13). Because Mac/IdeS cleaves IgG (17) and treatment of human blood or serum with recombinant Mac/IdeS protein prevented GAS opsonophagocytosis (4, 17, 19), it was hypothesized that Mac contributes to the invasive and highly virulent disease phenotype of this pre-eminent GAS strain. Furthermore, Mac could potentially protect invasive bacteria in tissues and the bloodstream, where IgG levels are high and Ig-Fc binding proteins do not protect bacteria from opsonophagocytosis (7). While Mac/IdeS expression, at both the transcript and protein levels, was markedly increased in the M1T1 AP strain (Fig. 1), we found no significant difference in the susceptibility of a *mac* knockout strain to whole blood, neutrophil, or macrophage killing and no loss of systemic virulence potential in naive or GAS-immunized mice (Fig. 2 and 3). While this study did not quantify the amount of Mac protease produced by the bacteria, the bacteria produced sufficiently detectable amounts of protein (Fig. 1B) with qualitatively corresponding IgG protease activity (Fig. 1 and 2A). Thus, in the AP M1T1 strain and within the limitations of our *in vitro* system, Mac/IdeS fails to satisfy molecular Koch's postulates when either expressed at physiologic concentrations (WT) or overexpressed (complemented strains) by GAS, and in the full context of multiple upregulated virulence genes coinciding with the shift to invasive infection, Mac/IdeS has no significant impact on GAS phagocyte resistance and mouse pathogenicity.

Though our experimental model systems used gold standard assays, the experiments are only proxies in assessing virulence, and differences from real infections could account for a lack of phenotype in our mutant strain. For example, Mac/IdeS-specific antibodies in these experiments could neutralize the activity or function of the protein. However, as we observed clear evidence of IgG cleavage in the donor serum containing GAS antibodies (Fig. 1C and 2A), the effects of neutralizing antibodies appear minimal, if not absent. IgG is the only described substrate for Mac/IdeS (21), and no role for Mac/IdeS in resistance to other phagocyte bactericidal mechanisms independent of antibody-mediated opsonophagocytosis, including killing by the THP-1 cell line lacking FcγRIII (Fig. 2D) or by the antimicrobial peptide LL-37 (Table 1) could be established in our *in vitro* systems. However, this does not preclude the activity of the enzyme on other as-yet-undiscovered substrates that may play a role in bacterial virulence.

With an abundance of phagocyte resistance factors, functional redundancy in the M1T1 clone may preclude the clear demonstration of a hidden human Mac/IdeS virulence role in *ex vivo* assays or the mouse model. However, analyses of a series of similarly constructed mutants of the same parent M1T1 strain (5448) have demonstrated clear phagocyte resistance and mouse virulence phenotypes for DNase Sda1 (26), M protein (30), streptolysin O (31), streptolysin S (32), hyaluronic acid capsule (16), SIC (27), and IL-8 peptidase SpyCEP (33), indicating that any independent Mac/IdeS contribution to virulence is likely minor in comparison. These studies demonstrate the utility of mutagenesis studies of the living pathogen to corroborate potential virulence functions attributed to bacterial proteins solely on the basis of biochemical studies or assays with recombinant forms at arbitrary concentrations.

Because Mac is a secreted protein and IgG is one of the most abundant molecules in serum besides albumin, a protective role for Mac/IdeS in the bacteria could potentially be limited because of dilution of the enzyme in the cellular and circulatory milieu and nonpreference of the enzyme for anti-GAS antibodies over the complete IgG pool. However, previous research has shown that all strains of GAS express either one of two alleles of the *mac*/*ideS* gene (complex I or complex II) and that each allele encodes an active protease with similar activity (19, 21, 34). Furthermore, other streptococcal species similarly encode a Mac-like protein (21). Evolutionary persistence and preservation of this gene indicate that the gene plays an important role in the overall ecology of the organism, perhaps in GAS colonization or transmission, or in localized mucosal infections such as pharyngitis and impetigo that are extremely challenging to model in the mouse. It is possible that in strains other than the M1T1 clone, Mac/IdeS could play a more prominent role in infection and resistance of the bacteria to the

host immune response. Local accumulation or high levels of expression of Mac/IdeS in these other GAS strains that do not express as many other virulence factors may contribute to GAS pathogenicity. Alternatively, and not mutually exclusively, the protease could also have other as-yet-unidentified substrates that would otherwise promote bacterial clearance.

MATERIALS AND METHODS

Cells and culture conditions. THP-1 (ATCC TIB-202) cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen). Whole blood was collected from healthy donors (use and procedures were approved by the University of California, San Diego [UCSD], Human Research Protections Program), and neutrophils were isolated with the PolyMorphPrep kit (Fresenius Kabi) as previously described (35).

Bacterial strains and mutant construction. The WT M1T1 GAS strain (5448) was originally isolated from a patient with necrotizing fasciitis and toxic shock syndrome (36) and maintained in Todd-Hewitt broth (THB) at 37°C. A precise, in-frame allelic replacement of *mac*/*ideS* with the chloramphenicol transferase (*cat*) gene was created in M1T1 GAS strain 5448 by a method previously described (26–28). The primer sets used to PCR amplify DNA fragments directly upstream and downstream of *mac*/*ideS* from the M1T1 GAS genome were mac-downF (5′ AAGAAT ACCTTTTTCTATAT 3′ with a 30-bp 5′ extension matching the 5′ end of the *cat* gene) plus mac-downR (5′ AGTAGCCCCTAAACTACTAT 3′) and mac-upF (5′ CGCTTGTTCTAAGTCTTCTAGTG 3′) plus mac-upR (5′ AGCGTATCAATAAGAAAGATTACC 3′ with a 30-bp 5′ extension matching the 3′ end of the *cat* gene). The mutant strain was verified by PCR and sequencing. An AP version of the M1T1 GAS parent strain (5448 AP) has been previously published and has been shown to contain a single inactivating adenine insertion at the 877-bp position of *covS* (14, 15). The AP version of the *mac* mutant strain was created by injecting 12-week-old male C57BL/6J mice (Charles River Laboratories) subcutaneously with 2×10^8 CFU of the mid-log-phase *mac* mutant strain in 100 μl Dulbecco's phosphate-buffered saline (PBS). After 72 h, the mice were sacrificed, lesions were excised and homogenized, and bacteria were plated. Bacteria were subsequently screened with a cysteine protease assay to detect SpeB activity (16), and the *covRS* genes of SpeB-negative colonies were sequenced. An 8-bp insertion at position 1212, producing a frameshift in the *covS* gene, was discovered. The AP mutant strain was designated the Δ*mac* mutant.

Plasmid construction and heterologous expression. The *mac*/*ideS* gene was amplified from the M1T1 genome by PCR with primers macF (5′ TTAATTGGTCTGATTCCAACTATC 3′) and macR (5′ ATGAGAA AAAGATGCTATTCAAC 3′). The PCR product was TA cloned into pCR2.1-TOPO (Invitrogen) and then subcloned into shuttle expression vector pDCerm (37). The resulting plasmid was introduced via electroporation into the Δ*mac* mutant and maintained in THB supplemented with 2 μg/ml erythromycin. The F129I point mutation predicted to disrupt proposed dimer formation (18) was generated by two-step PCR amplification of the *mac*/*ideS* gene in pCR2.1-TOPO with primers macF129IF (5′ GCAAAAAATAAACATCAATGGCGAACAGATG 3′) and macF and primers macF129IR (5′ CATCTGTTCGCCATTGATGTTTATTTTTTG C 3′) and macR. The product was gel purified and used as the template for a fusion PCR with the macF and macR primers to generate the final product. The final product was cloned into pCR2.1-TOPO (Invitrogen), and the point mutation was verified by sequencing. The gene was subcloned into shuttle expression vector pDCerm, and the resulting plasmid was likewise introduced via electroporation into the Δ*mac* mutant and maintained with 2-μg/ml erythromycin selection.

Real-time PCR. The WT and AP M1T1 GAS strains were grown to the log and stationary phases in THB. Approximately 1×10^9 to 2×10^9 CFU were washed two times with PBS and resuspended in RLT buffer (Qiagen) with β-mercaptoethanol. Bacterial cells were lysed by bead homogenization, and total RNA was isolated (Qiagen). Samples were DNase treated in accordance with the rigorous protocol in the manufacturer's instructions (Turbo DNase kit; Ambion). cDNA was synthesized from 250 to 500 ng RNA with the iScript kit (Bio-Rad), and real-time PCR was performed with iQ SYBR green Supermix (Bio-Rad) with primers Mac-fwd (5′ AGA GTTAACCGAAGGCAAGGCTCT 3′) and Mac-rev (5′ TCCCGTTAGA ATCAAAGTCAGCTCCC 3′), primers SpeB-fwd (5′ GTCGGTAAAGTA GGCGGACA 3′) and SpeB-rev (5′ GCCACCAGTACCAAGAGCT 3′), and primers gyrase A-fwd (5′ GAAGTGATCCCTGGACCTGA 3′) and gyrase A-rev (5′ CCCGACCTGTTTGAGTTGTT 3′). Data were normalized to gyrase A expression, and expression was calculated relative to that of log-phase WT M1T1 GAS.

Western blot analysis of Mac/IdeS. Culture supernatants of the indicated strains of log-phase (optical density at 600 nm [OD_{600}] of 0.4) and stationary-phase (OD_{600} of 1) bacteria were collected by pelleting at $13,000 \times g$ for 5 min. Culture supernatants were then filter sterilized with Spin-X columns with 0.22-μm filters (Corning). WT M1T1 culture supernatants were additionally concentrated fourfold in spin columns with a 10,000-Da molecular mass cutoff (Millipore). A 15-μl volume of culture supernatant from each sample was subjected to 12% SDS-PAGE, blotted, and probed with 1:1,000 anti-Mac/IdeS rabbit antiserum (22), followed by 1:15,000 goat anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (GE Healthcare). A single band at approximately 30 to 33 kDa was detected.

IgG cleavage assays. Cleavage assays of IgG from pooled human serum or purified human IgG (Sigma) were performed as described previously (17). Briefly, culture supernatants from log-phase bacterial cultures (OD_{600} of 0.4) were spun at $13,000 \times g$ for 5 min and subsequently filter sterilized with Spin-X columns with 0.22-μm filters (Corning). A 25-μl volume of supernatant was incubated with a final concentration of 5% pooled serum or 5 μg IgG in 100 μl (final volume) PBS. Samples were incubated for 30 min at 37°C, and 10 μl (1/10) of the reaction mixture was subsequently subjected to 12% SDS-PAGE. Gels were blotted and probed with 1:15,000 goat anti-human IgG HRP-conjugated antibodies (Promega).

Pooled serum collection and determination of donor serum titers. Blood from healthy human donors was collected in glass venous blood collection tubes with no additives (BD Biosciences) and allowed to clot at room temperature for 15 min. Blood was centrifuged at 4,000 rpm for 10 min at 4°C. The serum fraction was collected, aliquoted, and stored at −80°C. Serum was pooled from at least five individual donors. To heat inactivate the serum, aliquots were incubated at 56°C for 30 min. ELISAs to determine serum titers of antibody against the AP M1T1 strain at the log (OD_{600} of 0.4) and stationary (OD_{600} of 1) phases were performed twice as previously described (28).

Cell-killing assays. Bacterial strains from overnight cultures were inoculated into fresh THB with 5% pooled normal or heat-inactivated human serum and grown to log phase (OD_{600} of 0.4). For whole-blood assays, bacteria were diluted without washing to a final inoculum of 10^4 CFU, added to 300 μl freshly drawn blood, and rotated at 37°C. At 1 h postinfection, an aliquot of blood was removed, blood cells were lysed with water, and bacteria were enumerated on Todd-Hewitt agar (THA) plates. Experiments were performed with blood from three individual donors, and the pooled data are shown. For neutrophil assays, cells were stimulated with 25 nM phorbol 12-myristate 13-acetate (PMA; Sigma) for 20 min prior to the addition of bacteria. For THP-1 assays, cells were seeded 1 day prior into 24-well plates at 5×10^5 cells per well with a final concentration of 10 nM PMA to differentiate the cells to macrophages (38). THP-1 cells were washed once with PBS prior to the addition of bacteria. Bacteria grown in serum were added at a multiplicity of infection (MOI) of 0.1 bacterium per cell in RPMI supplemented with 2% 70°C heat-inactivated FBS (39) to neutrophils and THP-1 cells. Cells were spun to initiate bacterial contact, incubated at 37°C in 5% CO_2 for 90 min, lysed with a final concentration of 0.025% Triton X-100, diluted, and then enumerated by CFU counting on THA plates. The growth index was calculated as the ratio of surviving CFU after incubation compared with the

initial inoculum. For neutrophil assays, experiments were performed with blood from three individual donors, and pooled data are shown. For THP-1 assays, experiments were performed at least three times and pooled data are shown.

Phagocytosis assay. Bacterial strains were inoculated from overnight cultures into fresh THB with 5% normal human serum and grown to log phase (OD_{600} of 0.4). Bacteria were labeled by incubation for 30 min on ice in 0.2 mg/ml fluorescein isothiocyanate (FITC; Sigma). Neutrophils were stimulated with PMA for 15 min, followed by 20 min in the presence or absence of (10 μg/ml) cytochalasin D (Sigma) to prevent phagocytosis. Prepared bacteria were then added at an MOI of 1 to cells, and cultures were spun to initiate bacterial contact and incubated at 37°C in 5% CO_2 for 20 min. Antibiotics were added to kill extracellular bacteria. Cells were washed and analyzed by flow cytometry. The percentage of neutrophils containing phagocytosed bacteria was calculated by subtracting the FITC-positive signal from cytochalasin D-treated cells. Flow cytometry data were analyzed with FlowJo v. 9.4.10 (Tree Star, Inc.).

Oxidative-burst assay. Oxidative-burst assays were performed as previously described (40). Briefly, human neutrophils were resuspended to 2×10^6/ml in Hanks balanced salt solution (HBSS; Cellgro) without calcium and magnesium and incubated with 100 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 20 min. As a negative control, neutrophils were prepared simultaneously without DCFH-DA. Neutrophils were washed in HBSS without calcium and magnesium and resuspended to 5×10^6/ml in HBSS supplemented with calcium and magnesium, 5×10^5 cells/well were seeded into the wells of 96-well plates, and neutrophils were infected at an MOI of 1. The fluorescence intensity was read every 15 min with a SpectraMax M3 fluorescent plate reader at a 485-nm excitation wavelength and a 530-nm emission wavelength. Between readings, the cells were incubated in a horizontal shaker at 37°C and 80 rpm.

LL-37 MIC and MBC assays. A final concentration of 1 to 16 μM LL-37 (AnaSpec, EGT) was tested in RPMI plus 20% THB in 96-well plates. Log-phase bacteria were adjusted to a final concentration of 10^5 CFU/well. As a positive control, bacteria were incubated in medium alone. The plate was incubated for 24 h at 37°C, and the absorbance of the samples at 600 nm was read with a spectrophotometric plate reader. The MBC was measured by stamping the 24-h-incubated samples onto THA plates, and colony growth was scored after incubation overnight at 37°C.

Mouse immunization and infection models. All animal use and procedures were approved by the UCSD Institutional Animal Care and Use Committee. Immunization procedures were performed as previously described (5). Briefly, 10-week-old male CD-1 mice (Charles River Laboratories) were injected intraperitoneally with 2×10^6 to 4×10^6 CFU heat-killed WT M1T1 GAS in PBS on days 0, 7, and 14. Serum was collected from each mouse at days 0 (preimmune), 7 (bleed 1), 14 (bleed 2), and 28 (bleed 3), and serum samples from four mice (cagemates) were pooled. Five pooled samples from each collection day were tested by ELISA as previously described (28) with a rabbit anti-mouse HRP-conjugated secondary antibody at 1:5,000 (GE Healthcare). Mice were confirmed to have GAS-positive titers before the infection experiment.

Nonimmunized or day 35 postimmunization mice were infected subcutaneously with 4×10^7 to 5×10^7 CFU of the AP or Δ*mac* mutant strain. Survival was monitored every 12 h. Ten mice were used for each bacterial strain.

Statistical analysis. All real-time PCR and cell assay data were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison posttest. Oxidative-burst data were analyzed by two-way ANOVA. The mouse survival data were assessed by the log rank (Mantel-Cox) test. All statistical tests were performed with GraphPad Prism version 5.0 (GraphPad Software Inc.). *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00499-13/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB
Figure S1, TIF file, 2.5 MB.
Figure S2, TIF file, 2.4 MB.

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