

# 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.

Received 4 July 2013 Accepted 9 July 2013 Published 30 July 2013

**Citation** Okumura CYM, Anderson EL, Döhrmann S, Tran DN, Olson J, von Pawel-Rammingen U, Nizet V. 2013. IgG protease Mac/IdeS is not essential for phagocyte resistance or mouse virulence of M1T1 group A *Streptococcus*. *mBio* 4(4):e00499-13. doi:10.1128/mBio.00499-13.

**Editor** Michael Gilmore, Harvard Medical School

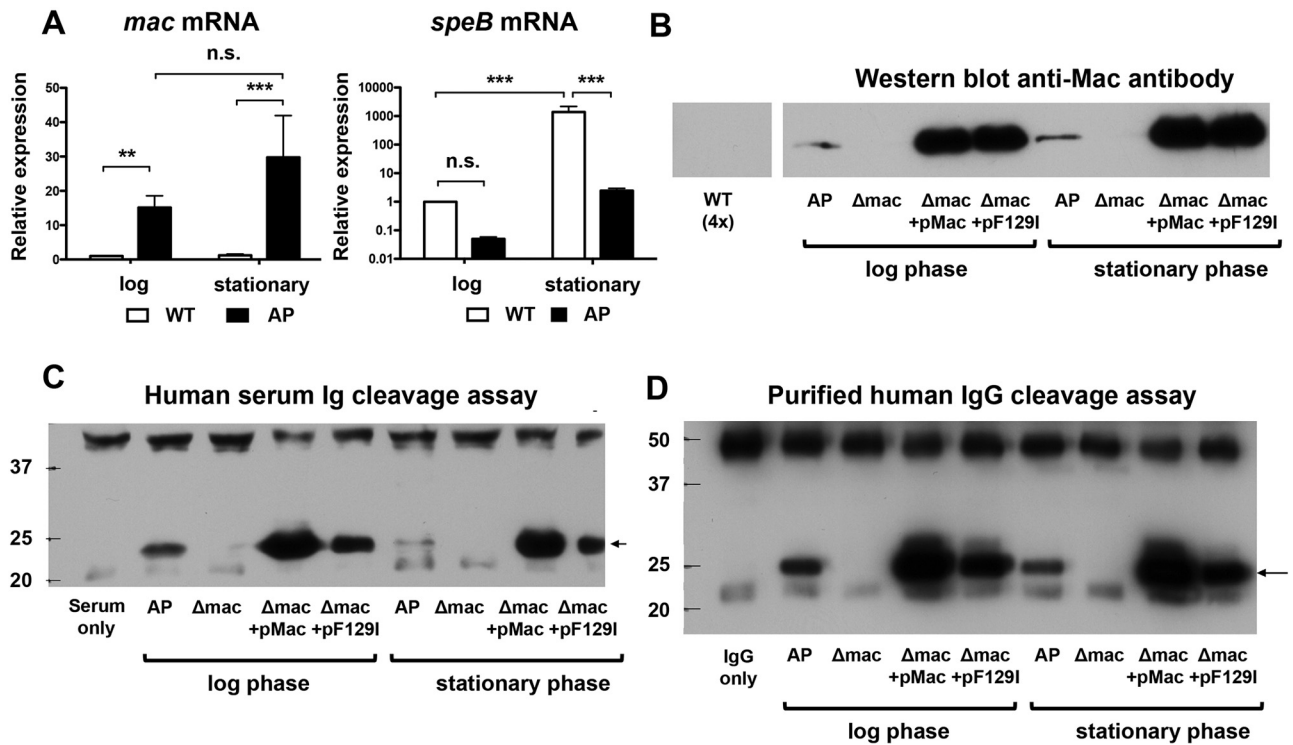
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The Gram-positive bacterium group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is responsible for a broad spectrum of diseases, ranging from the relatively mild pathologies of pharyngitis and impetigo to severe invasive diseases such as necrotizing 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 phenotypic 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 MIT1 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 MIT1 GAS compared to the original WT strain prior to AP. Data are shown as the mean  $\pm$  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 MIT1 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 MIT1 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,  $\Delta mac$  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).

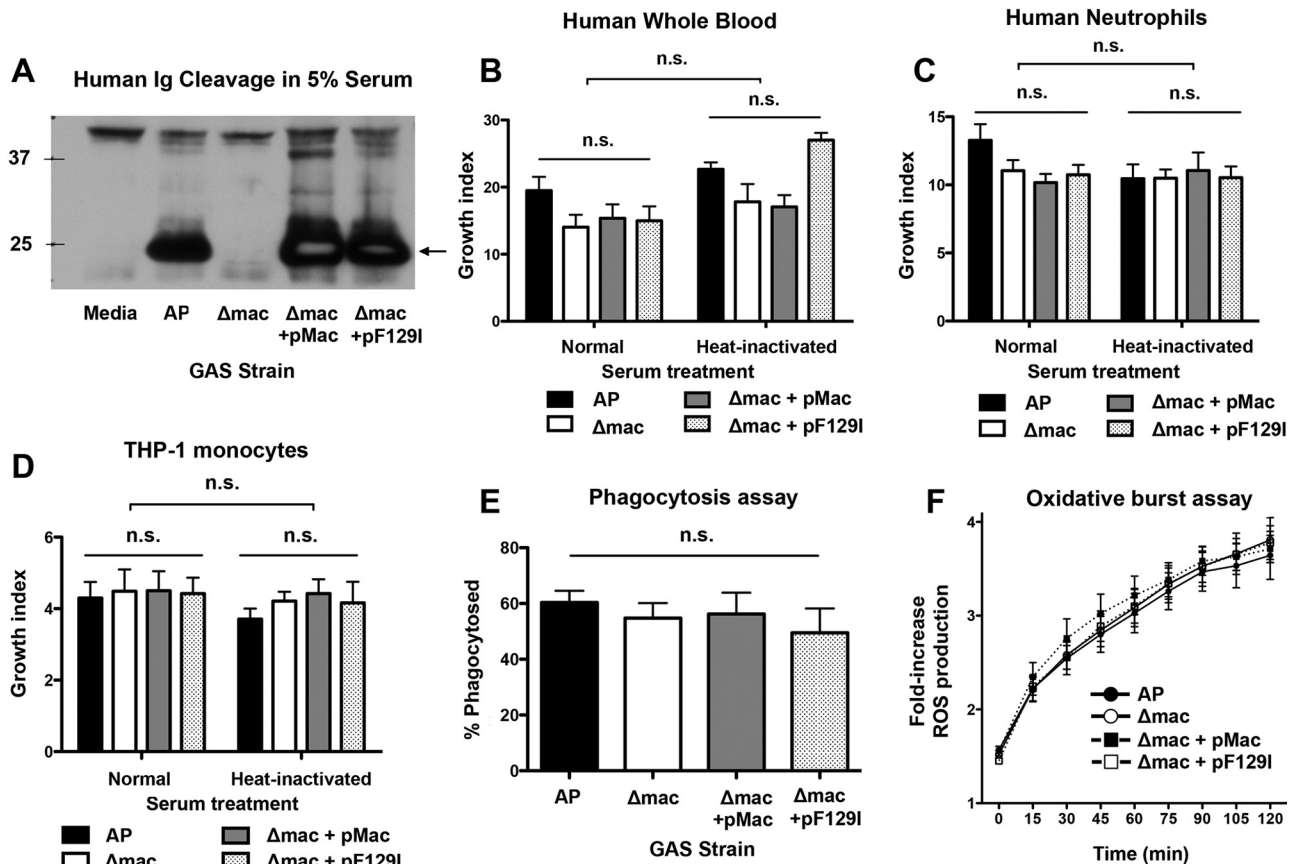
One of the highly upregulated genes in the MIT1 clone associated with the *covRS* switch is that encoding the Mac protein (also designated IgG-degrading enzyme of *S. pyogenes*, IdeS) (13), a secreted 35-kDa protein with cleavage specificity for the hinge region of the IgG heavy chain (17). The name Mac derives from limited sequence homology with the  $\alpha$  subunit of human Mac-1 (CD-11), a  $\beta 2$  integrin (4), and its ability to bind  $\alpha v \beta 3$  and  $\alpha IIb \beta 3$  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-opsinized 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 MIT1 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 MIT1 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 MIT1 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 MIT1 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  $\Delta mac$  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,  $\Delta mac$  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,  $\Delta mac$  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  $\pm$  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 sub-

cutaneous 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  $\Delta mac$  mutant strain. For complementation analysis, *mac/ideS* was cloned into an expression plasmid (pMac) and transformed into the  $\Delta mac$  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  $\Delta mac$  mutant strain. Mac protein was absent from the  $\Delta mac$  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,  $\Delta mac$  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  $\Delta mac$  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,  $\Delta mac$  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 oxida-

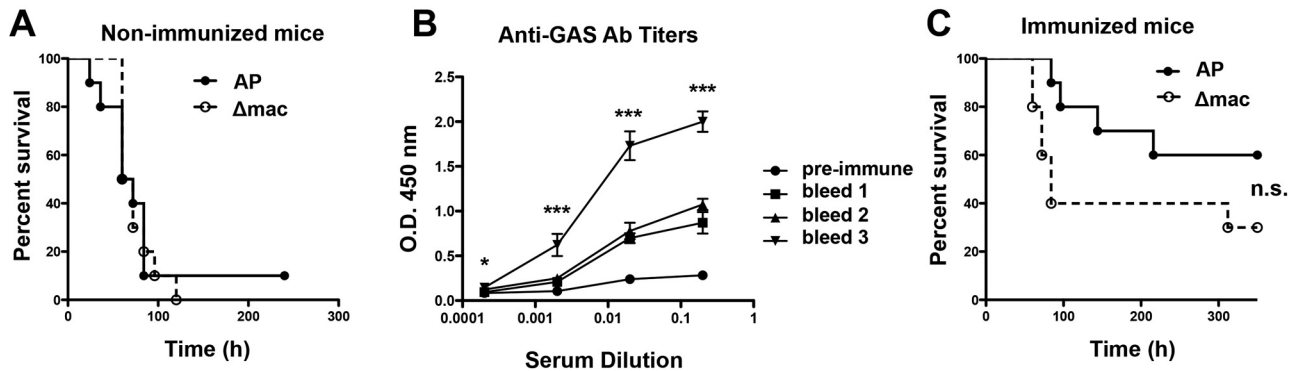
TABLE 1 LL-37 MICs and MBCs for the strains used in this study<sup>a</sup>

Strain	MIC ( $\mu\text{M}$ )	MBC ( $\mu\text{M}$ )
AP	8–16	8–16
$\Delta mac$ mutant	8–16	8–16
$\Delta mac$ mutant/pMac	8–16	8–16
$\Delta mac$ mutant/pF129I	8–16	8–16

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

tive 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,  $\Delta mac$  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.

**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  $\Delta mac$  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  $\Delta mac$  mutant were noted (Fig. 3A), indicating that in a GAS-naïve 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  $\Delta mac$  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 MIT1 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  $\Delta mac$  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  $\Delta mac$  mutant strain. For each panel, 10 mice were used per strain. In panel B, data are shown as the mean  $\pm$  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 MIT1, 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 MIT1 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 MIT1 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 Fc $\gamma$ RIII (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 MIT1 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 MIT1 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 MIT1 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 MIT1 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 MIT1 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 MIT1 GAS genome were mac-downF (5' AAGAATACCTTTTCTATAT 3' with a 30-bp 5' extension matching the 5' end of the *cat* gene) plus mac-downR (5' AGTAGCCCCCTAACTACTAT 3') and mac-upF (5' CGCTGTCTAAGTCTTCTAGTG 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 MIT1 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  $2 \times 10^8$  CFU of the mid-log-phase *mac* mutant strain in 100  $\mu$ l 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  $\Delta mac$  mutant.

**Plasmid construction and heterologous expression.** The *mac/ideS* gene was amplified from the MIT1 GAS genome by PCR with primers macF (5' TTAATTGGTCTGATTCCAACACTATC 3') and macR (5' ATGAGAA AAGATGCTATTCAAC 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  $\Delta mac$  mutant and maintained in THB supplemented with 2  $\mu$ g/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' CATCTGTTCCGACATGATGTTATTTTTTTC 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  $\Delta mac$  mutant and maintained with 2- $\mu$ g/ml erythromycin selection.

**Real-time PCR.** The WT and AP MIT1 GAS strains were grown to the log and stationary phases in THB. Approximately  $1 \times 10^9$  to  $2 \times 10^9$  CFU were washed two times with PBS and resuspended in RLT buffer (Qiagen) with  $\beta$ -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 ATCAAAGTCAGTCCC 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' CCCGACCTGTTGAGTTGTT 3'). Data were normalized to gyrase A expression, and expression was calculated relative to that of log-phase WT MIT1 GAS.

**Western blot analysis of Mac/IdeS.** Culture supernatants of the indicated strains of log-phase (optical density at 600 nm [OD<sub>600</sub>] of 0.4) and stationary-phase (OD<sub>600</sub> 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- $\mu$ m filters (Corning). WT MIT1 culture supernatants were additionally concentrated fourfold in spin columns with a 10,000-Da molecular mass cutoff (Millipore). A 15- $\mu$ l 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<sub>600</sub> of 0.4) were spun at 13,000  $\times g$  for 5 min and subsequently filter sterilized with Spin-X columns with 0.22- $\mu$ m filters (Corning). A 25- $\mu$ l volume of supernatant was incubated with a final concentration of 5% pooled serum or 5  $\mu$ g IgG in 100  $\mu$ l (final volume) PBS. Samples were incubated for 30 min at 37°C, and 10  $\mu$ l (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 MIT1 strain at the log (OD<sub>600</sub> of 0.4) and stationary (OD<sub>600</sub> 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<sub>600</sub> of 0.4). For whole-blood assays, bacteria were diluted without washing to a final inoculum of  $10^4$  CFU, added to 300  $\mu$ l 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  $5 \times 10^5$  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<sub>2</sub> 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  $\mu$ g/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  $2 \times 10^6$ /ml in Hanks balanced salt solution (HBSS; Cellgro) without calcium and magnesium and incubated with 100  $\mu$ M 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  $5 \times 10^6$ /ml in HBSS supplemented with calcium and magnesium,  $5 \times 10^5$  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  $\mu$ M 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  $2 \times 10^6$  to  $4 \times 10^6$  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  $4 \times 10^7$  to  $5 \times 10^7$  CFU of the AP or  $\Delta mac$  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.

## ACKNOWLEDGMENTS

Support for this work was provided by National Institutes of Health grants AI077780 and AR052728 to V.N., the UCSD/SDSU IRACDA Postdoctoral Fellowship Program (GM068524) to C.O., Swedish Research Council project 2009-4997 to U.V.P.-R., the Deutschlandstipendium of the Technische Universität Braunschweig to S.D., and a Diversity Supplement (AI077780) to E.A.

We thank Anjuli Timmer and Suzan Rooijackers for their technical advice.

## REFERENCES

- Carapetis JR, Steer AC, Mulholland EK, Weber M. 2005. The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* 5:685–694.
- Cunningham MW. 2000. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* 13:470–511.
- Akesson P, Rasmussen M, Mascini E, von Pawel-Rammingen U, Janulczyk R, Collin M, Olsen A, Mattsson E, Olsson ML, Björck L, Christensson B. 2004. Low antibody levels against cell wall-attached proteins of *Streptococcus pyogenes* predispose for severe invasive disease. *J. Infect. Dis.* 189:797–804.
- Lei B, DeLeo FR, Hoe NP, Graham MR, Mackie SM, Cole RL, Liu M, Hill HR, Low DE, Federle MJ, Scott JR, Musser JM. 2001. Evasion of human innate and acquired immunity by a bacterial homolog of CD11b that inhibits opsonophagocytosis. *Nat. Med.* 7:1298–1305.
- Siebert J, Sastalla I, Chhatwal GS, Medina E. 2006. Vaccination equally enables both genetically susceptible and resistant mice to control infection with group A streptococci. *Microbes Infect.* 8:347–353.
- Johansson L, Thulin P, Low DE, Norrby-Teglund A. 2010. Getting under the skin: the immunopathogenesis of *Streptococcus pyogenes* deep tissue infections. *Clin. Infect. Dis.* 51:58–65.
- Nordenfelt P, Waldemarson S, Linder A, Mörgelin M, Karlsson C, Malmström J, Björck L. 2012. Antibody orientation at bacterial surfaces is related to invasive infection. *J. Exp. Med.* 209:2367–2381.
- Cole JN, Barnett TC, Nizet V, Walker MJ. 2011. Molecular insight into invasive group A streptococcal disease. *Nat. Rev. Microbiol.* 9:724–736.
- Kwinn LA, Nizet V. 2007. How group A *Streptococcus* circumvents host phagocyte defenses. *Future Microbiol.* 2:75–84.
- Nizet V. 2007. Understanding how leading bacterial pathogens subvert innate immunity to reveal novel therapeutic targets. *J. Allergy Clin. Immunol.* 120:13–22.
- Aziz RK, Kotb M. 2008. Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. *Emerg. Infect. Dis.* 14:1511–1517.
- Maamary PG, Ben Zakour NL, Cole JN, Hollands A, Aziz RK, Barnett TC, Cork AJ, Henningham A, Sanderson-Smith M, McArthur JD, Venturini C, Gillen CM, Kirk JK, Johnson DR, Taylor WL, Kaplan EL, Kotb M, Nizet V, Beatson SA, Walker MJ. 2012. Tracing the evolutionary history of the pandemic group A streptococcal M1T1 clone. *FASEB J.* 26:4675–4684.
- Sumby P, Whitney AR, Graviss EA, DeLeo FR, Musser JM. 2006. Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathog.* 2:e5. doi:10.1371/journal.ppat.0020005.
- Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, Simpson AJ, Buchanan JT, Chhatwal GS, Kotb M, Nizet V. 2007. DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nat. Med.* 13:981–985.
- Aziz RK, Pabst MJ, Jeng A, Kansal R, Low DE, Nizet V, Kotb M. 2004. Invasive M1T1 group A streptococcus undergoes a phase-shift in vivo to prevent proteolytic degradation of multiple virulence factors by SpeB. *Mol. Microbiol.* 51:123–134.
- Cole JN, Pence MA, von Kockritz-Blickwede M, Hollands A, Gallo RL, Walker MJ, Nizet V. 2010. M protein and hyaluronic acid capsule are essential for *in vivo* selection of *covRS* mutations characteristic of invasive serotype M1T1 group A *Streptococcus*. *mBio* 1:e00191-10. doi:10.1128/mBio.00191-10.
- von Pawel-Rammingen U, Johansson BP, Björck L. 2002. IdeS, a novel

- streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* 21:1607–1615.
18. Agniswamy J, Nagiec MJ, Liu M, Schuck P, Musser JM, Sun PD. 2006. Crystal structure of group A *Streptococcus* Mac-1: insight into dimer-mediated specificity for recognition of human IgG. *Structure* 14:225–235.
  19. Lei B, DeLeo FR, Reid SD, Voyich JM, Magoun L, Liu M, Braughton KR, Ricklefs S, Hoe NP, Cole RL, Leong JM, Musser JM. 2002. Opsonophagocytosis-inhibiting Mac protein of group A *Streptococcus*: identification and characteristics of two genetic complexes. *Infect. Immun.* 70:6880–6890.
  20. Vindebro R, Spoerry C, von Pawel-Rammingen U. 2013. Rapid IgG heavy chain cleavage by the streptococcal IgG endopeptidase IdeS is mediated by IdeS monomers and is not due to enzyme dimerization. *FEBS Lett.* 587:1818–1822.
  21. von Pawel-Rammingen U. 2012. Streptococcal IdeS and its impact on immune response and inflammation. *J. Innate Immun.* 4:132–140.
  22. Söderberg JJ, von Pawel-Rammingen U. 2008. The streptococcal protease IdeS modulates bacterial IgGfC binding and generates 1/2Fc fragments with the ability to prime polymorphonuclear leucocytes. *Mol. Immunol.* 45:3347–3353.
  23. Collin M, Olsén A. 2001. Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect. Immun.* 69:7187–7189.
  24. Collin M, Olsén A. 2001. EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *EMBO J.* 20:3046–3055.
  25. Persson H, Vindebro R, von Pawel-Rammingen U. 2013. The streptococcal cysteine protease SpeB is not a natural immunoglobulin cleaving enzyme. *Infect. Immun.* 81:2236–2241.
  26. Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, Feramisco J, Nizet V. 2006. DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr. Biol.* 16:396–400.
  27. Pence MA, Rooijackers SH, Cogen AL, Cole JN, Hollands A, Gallo RL, Nizet V. 2010. Streptococcal inhibitor of complement promotes innate immune resistance phenotypes of invasive M1T1 group A *Streptococcus*. *J. Innate Immun.* 2:587–595.
  28. Sjögren J, Okumura CY, Collin M, Nizet V, Hollands A. 2011. Study of the IgG endoglycosidase EndoS in group A streptococcal phagocyte resistance and virulence. *BMC Microbiol.* 11:120. doi:10.1186/1471-2180-11-120.
  29. Nandakumar KS, Johansson BP, Björck L, Holmdahl R. 2007. Blocking of experimental arthritis by cleavage of IgG antibodies in vivo. *Arthritis Rheum.* 56:3253–3260.
  30. Lauth X, von Köckritz-Blickwede M, McNamara CW, Myskowski S, Zinkernagel AS, Beall B, Ghosh P, Gallo RL, Nizet V. 2009. M1 protein allows group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. *J. Innate Immun.* 1:202–214.
  31. Timmer AM, Timmer JC, Pence MA, Hsu LC, Ghochani M, Frey TG, Karin M, Salvesen GS, Nizet V. 2009. Streptolysin O promotes group A *Streptococcus* immune evasion by accelerated macrophage apoptosis. *J. Biol. Chem.* 284:862–871.
  32. Datta V, Myskowski SM, Kwinn LA, Chiem DN, Varki N, Kansal RG, Kotb M, Nizet V. 2005. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Mol. Microbiol.* 56:681–695.
  33. Zinkernagel AS, Timmer AM, Pence MA, Locke JB, Buchanan JT, Turner CE, Mishalian I, Sriskandan S, Hanski E, Nizet V. 2008. The IL-8 protease SpyCEP/ScpC of group A *Streptococcus* promotes resistance to neutrophil killing. *Cell Host Microbe* 4:170–178.
  34. Söderberg JJ, Engström P, von Pawel-Rammingen U. 2008. The intrinsic immunoglobulin G endopeptidase activity of streptococcal Mac-2 proteins implies a unique role for the enzymatically impaired Mac-2 protein of M28 serotype strains. *Infect. Immun.* 76:2183–2188.
  35. Okumura CY, Hollands A, Tran DN, Olson J, Daresh S, von Köckritz-Blickwede M, Thienphrapa W, Corle C, Jeung SN, Kotsakis A, Shalwitz RA, Johnson RS, Nizet V. 2012. A new pharmacological agent (AKB-4924) stabilizes hypoxia inducible factor-1 (HIF-1) and increases skin innate defenses against bacterial infection. *J. Mol. Med.* 90:1079–1089.
  36. Chatellier S, Ihendyane N, Kansal RG, Khambaty F, Basma H, Norrby-Teglund A, Low DE, McGeer A, Kotb M. 2000. Genetic relatedness and superantigen expression in group A *Streptococcus* serotype M1 isolates from patients with severe and nonsevere invasive diseases. *Infect. Immun.* 68:3523–3534.
  37. Jeng A, Sakota V, Li Z, Datta V, Beall B, Nizet V. 2003. Molecular genetic analysis of a group A *Streptococcus* operon encoding serum opacity factor and a novel fibronectin-binding protein, SfbX. *J. Bacteriol.* 185:1208–1217.
  38. Park EK, Jung HS, Yang HI, Yoo MC, Kim C, Kim KS. 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.* 56:45–50.
  39. von Köckritz-Blickwede M, Chow OA, Nizet V. 2009. Fetal calf serum contains heat-stable nucleases that degrade neutrophil extracellular traps. *Blood* 114:5245–5246.
  40. Chow OA, von Köckritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, Gallo RL, Monestier M, Wang Y, Glass CK, Nizet V. 2010. Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe* 8:445–454.