

The Fibrinogen-binding M1 Protein Reduces Pharyngeal Cell Adherence and Colonization Phenotypes of M1T1 Group A *Streptococcus**

Received for publication, October 22, 2013, and in revised form, December 16, 2013. Published, JBC Papers in Press, December 19, 2013, DOI 10.1074/jbc.M113.529537

Ericka L. Anderson[‡], Jason N. Cole^{‡§}, Joshua Olson[‡], Bryan Ryba[‡], Partho Ghosh[¶], and Victor Nizet^{¶||1}

From the Departments of [‡]Pediatrics and [¶]Chemistry and Biochemistry and ^{||}Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California 92093 and the [§]School of Chemistry and Molecular Biosciences and Australian Infectious Diseases Research Center, University of Queensland, St. Lucia, Queensland 4072, Australia

Background: The group A *Streptococcus* (GAS) M1 protein binds fibrinogen (Fg) to block phagocytosis and to form a proinflammatory complex.

Results: M1 and Fg limit GAS adherence and invasion of pharyngeal keratinocytes *in vitro*.

Conclusion: Protease SpeB modulates M1 expression and GAS host cell interactions differentially during the course of infection.

Significance: M1 protein is shown to impede pharyngeal colonization *in vivo*.

Group A *Streptococcus* (GAS) is a leading human pathogen producing a diverse array of infections from simple pharyngitis (“strep throat”) to invasive conditions, including necrotizing fasciitis and toxic shock syndrome. The surface-anchored GAS M1 protein is a classical virulence factor that promotes phagocyte resistance and exaggerated inflammation by binding host fibrinogen (Fg) to form supramolecular networks. In this study, we used a virulent WT M1T1 GAS strain and its isogenic M1-deficient mutant to examine the role of M1-Fg binding in a proximal step in GAS infection-interaction with the pharyngeal epithelium. Expression of the M1 protein reduced GAS adherence to human pharyngeal keratinocytes by 2-fold, and this difference was increased to 4-fold in the presence of Fg. In stationary phase, surface M1 protein cleavage by the GAS cysteine protease SpeB eliminated Fg binding and relieved its inhibitory effect on GAS pharyngeal cell adherence. In a mouse model of GAS colonization of nasal-associated lymphoid tissue, M1 protein expression was associated with an average 6-fold decreased GAS recovery in isogenic strain competition assays. Thus, GAS M1 protein-Fg binding reduces GAS pharyngeal cell adherence and colonization in a fashion that is counterbalanced by SpeB. Inactivation of SpeB during the shift to invasive GAS disease allows M1-Fg binding, increasing pathogen phagocyte resistance and proinflammatory activities.

The Gram-positive bacterium *Streptococcus pyogenes*, also known as group A *Streptococcus* (GAS),² is an important, exclu-

sively human pathogen that is responsible for many clinical diseases, ranging from simple pharyngitis (“strep throat”) to life-threatening invasive conditions such as necrotizing fasciitis and streptococcal toxic shock-like syndrome (1, 2). Furthermore, repeated GAS infection can provoke serious autoimmune sequelae, including acute glomerulonephritis, rheumatic fever, and rheumatic heart disease; the latter remains a leading cause of morbidity and mortality in many parts of the developing world (3). Although oral antibiotic therapy is effective for noninvasive GAS infections such as pharyngitis, severe invasive GAS diseases typically require aggressive supportive care and surgical interventions (4, 5). To date, a safe and efficacious GAS vaccine has yet to be developed (6, 7).

GAS strains are classified by serotype based on the M protein, an antigenically variable surface-anchored protein encoded by the *emm* gene (8, 9). M proteins possess a highly conserved C-terminal region that is required for covalent cell surface attachment and a highly variable N-terminal region that extends outwards from the cell wall (10, 11). M proteins form surface fibrils, with almost their entire length appearing to form a dimeric α -helical coiled coil (12). Serotype specificity occurs within the hypervariable N-terminal region of 40–50 amino acid residues, and >200 *emm* sequence types have been identified (13). Serotype M1 is consistently among the most commonly identified serotypes in streptococcal pharyngitis (14) and invasive diseases worldwide (3, 15). Since the 1980s, a marked worldwide resurgence in severe invasive GAS diseases, including streptococcal toxic shock-like syndrome and necrotizing fasciitis, has been correlated with the global dissemination of a single GAS serotype, the M1T1 clone (16, 17).

After colonization of the epithelium, the bacteria penetrate the subepithelial tissue, where neutrophils are recruited and exert a selective pressure to which the bacteria must adapt for the infection to proceed into the blood. This transition from localized to systemic infection by GAS serotype M1T1 involves spontaneous mutations within the CovRS two-component system, which regulates ~10% of the GAS genome, resulting in

* This work was supported, in whole or in part, by National Institutes of Health Grants AI096837 (to P. G. and V. N.) and AI077780 (to V. N.). This work was also supported by a UNCF/Merck postdoctoral fellowship (to E. L. A.) and Australian National Health and Medical Research Council Grant APP1033258 (to J. N. C.).

¹ To whom correspondence should be addressed: Dept. of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Dr., Mail Code 0687, La Jolla, CA 92093-0687. Tel.: 858-534-7408; Fax: 858-534-5611; E-mail: vnizet@ucsd.edu.

² The abbreviations used are: GAS, group A *Streptococcus*; Fg, fibrinogen; NALT, nasal-associated lymphoid tissue.

Fg-binding M1 Protein Reduces GAS Pharyngeal Colonization

strong transcriptional up-regulation of multiple virulence-associated genes (2, 18). The up-regulated genes include the operon for synthesis of the hyaluronic acid capsule and the genes encoding streptolysin O, NAD glycohydrolase, the interleukin-8 protease SpyCEP/ScpC, and the DNase Sda1. These mutations in the *covRS* operon also abolish expression of streptococcal pyrogenic exotoxin B (SpeB), a broad-spectrum, secreted cysteine protease (19) that can degrade several GAS extracellular proteins and virulence factors (20). Loss of SpeB protease activity prevents degradation of streptokinase (a plasminogen activator), the M1 surface protein, and host plasminogen, enabling GAS serotype M1T1 to accumulate cell surface plasmin activity via M1-bound fibrinogen (Fg) and promote invasive infection by enhancing bacterial dissemination (21).

M proteins mediate GAS resistance to opsonophagocytosis by binding Fg, complement inhibitor factor H, and C4BP (C4b-binding protein) to the GAS cell surface (22–24). Surface-bound Fg masks receptors for complement protein C3b, whereas factor H down-regulates the production of C3b, as does C4BP. Together, C3b deposition on the GAS cell surface and C3b interaction with neutrophils are blocked. Furthermore, after proteolytic release from the GAS surface, the M1 type of the M protein forms a proinflammatory supramolecular network with Fg that contributes to the pathophysiology of streptococcal toxic shock-like syndrome (25, 26).

A proximal step in the pathogenesis of GAS infection is colonization of the pharyngeal epithelium. Whereas M protein binding to Fg promotes immune evasion by reducing phagocytic cell interactions, the effect of this phenomenon on GAS interaction with epithelial cells has not yet been described. Colonization of epithelial cells is initially a weak interaction mediated by lipoteichoic acid or pili, followed by a stronger binding via lectin-carbohydrate and/or protein-protein interactions that confer tissue specificity. Fibronectin has been demonstrated to serve as a receptor for many GAS adhesion proteins (27). M proteins can interact with extracellular matrix proteins and therefore promote epithelial cell adherence (27–31). However, this function may depend on the M serotype and target cell; for example, M6 and M24 proteins mediate adherence of GAS to human soft palate, whereas M18 does not (32). These considerations led us to study how the M1 protein (and in particular, its ability to bind Fg) influences pharyngeal epithelial interactions during early GAS infection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

GAS serotype M1T1 isolate 5448 (WT) was originally isolated from a patient with necrotizing fasciitis and toxic shock (33). The isogenic in-frame allelic exchange knock-out mutants 5448 Δ *emm1* (Δ M1) (34) and 5448 Δ *speB* (Δ SpeB) (35) have been described previously. All GAS strains were routinely propagated at 37 °C on Todd-Hewitt agar (Difco) or in static liquid cultures of Todd-Hewitt broth. Where appropriate, strains were grown in medium supplemented with 5 μ g/ml erythromycin.

Assays for Bacterial Fg binding

Flow Assay—Fg (human plasminogen-depleted; Calbiochem) was labeled with FITC using the FluoReporter protein labeling kit (Invitrogen) and added at 100 μ g/ml to GAS strains grown to $A_{600} = 0.4$ in Todd-Hewitt broth. Samples were incubated for 30 min with rotating and washed with PBS prior to flow cytometric analysis. The percentage of cells bound by Fg was determined relative to the WT strain.

Plate-based Assay—Microtiter plates were coated overnight at 4 °C with 200 μ l of Fg at 0.2 mg/ml in 50 mM Na₂CO₃ (pH 9.5). The wells were washed three times with PBS and blocked with 1% bovine serum albumin for 2 h at 37 °C. Then, 100 μ l of 2×10^8 bacteria were added and spun at $500 \times g$ for 10 min. Plates were incubated at 37 °C for 1 h; the supernatant was removed with a pipette and washed five times with PBS; and 100 μ l of 0.25% trypsin + 1 mM EDTA were added. Plates were incubated at 37 °C for an additional 10 min, and 100 μ l of PBS was added, followed by trituration 50 times. Serial dilutions were plated onto Todd-Hewitt agar plates for cfu enumeration.

Fg-bound Inoculum—Log-phase group A streptococci were incubated with or without 8 μ g/ml Fg for 30 min, and serial dilutions were plated to enumerate cfu in the presence or absence of Fg.

Adherence and Invasion Assays, Including Fluorescence Microscopy

Adherence and invasion assays were performed as described previously (36) but using the HaCaT human skin keratinocyte cell line or the OKP7/tert pharyngeal keratinocyte cell line (37), a generous gift from the Harvard Skin Disease Research Center Cell Culture Core (J. Rheinwald, Director). OKP7 cells were first grown to 80% confluence in keratinocyte serum-free medium (Invitrogen) + 25 μ g/ml bovine pituitary extract + 0.2 ng/ml EGF + 0.3 mM Ca²⁺. The cell were then split into 24-well plates and cultivated in 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture + 0.2 ng/ml EGF + 25 μ g/ml bovine pituitary extract + 2 mM L-glutamine with or without 8 μ g/ml Fg. HaCaT cells were cultivated in RPMI 1640 medium + 10% FBS. GAS strains in the logarithmic growth phase ($A_{600} = 0.4$) or stationary phase (21-h growth) were resuspended in PBS and added to the keratinocyte cells at a multiplicity of infection of 10. Imaging analysis was performed using FITC-labeled GAS (30-min treatment with 0.2 mg/ml FITC, covered on ice, and washed with PBS). Images were taken using a Zeiss Axiovert 40 CFL microscope, 10 \times Achromplan or 32 \times Achromstigmat objectives, a Zeiss AxioCam MR camera, and AxioVision AC software and processed in Photoshop to overlap the FITC signal with the bright-field image.

Mouse Nasal-associated Lymphoid Tissue (NALT) Colonization Assay

Mice were inoculated as described previously (38). Briefly, GAS were grown to $A_{600} = 0.4$ and resuspended to 2×10^7 cfu/ μ l in PBS. GAS were mixed at a 1:1 ratio of WT to Δ M1 bacteria or complemented mutant to Δ M1 bacteria; 10 μ l of the 1:1 mixture were pipetted into each nostril (20 μ l/mouse = 4×10^8 cfu total) of 8–12-week-old C57BL/6 mice under anesthesia; the inoculum was allowed to dry. Mice were killed 24 h

post-infection; the NALT was dissected as described previously (38) and homogenized in PBS; and serial dilutions were plated onto blood agar, Todd-Hewitt agar + 5 $\mu\text{g}/\text{ml}$ erythromycin, or Todd-Hewitt agar + 2 $\mu\text{g}/\text{ml}$ chloramphenicol plates for enumeration of cfu. For histopathological studies, NALT was dissected, paraffin-sectioned and Gram-stained. Images were taken using a Motic BA400 microscope, a Motic 40 \times objective, a Moticam 2500 camera, and Motic Images Plus 2.0 software.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of California, San Diego (Animal Welfare Assurance A3033-01). All efforts were made to minimize the suffering of the animals employed in this study.

RESULTS

M1 Protein Is the Major Fg-binding Protein of MIT1 GAS Strain 5448—Previous studies have demonstrated the importance of Fg binding by surface-expressed M proteins in restricting complement deposition and blocking GAS opsonophagocytosis (22, 28, 39). In addition, recent work has shown that the contribution of the M1 protein to GAS systemic virulence is dependent on the serum concentration of Fg (40). As a first step in our analysis, we tested the contribution of the M1 protein to Fg binding by WT MIT1 GAS strain 5448, representative of the contemporary hypervirulent clone associated with pharyngitis and invasive infections worldwide. In a flow cytometry-based assay using fluorescently labeled Fg, WT MIT1 GAS had markedly higher levels of Fg binding than the isogenic ΔM1 mutant, and reintroduction of the M1 protein on a plasmid vector to the ΔM1 mutant strain (complemented mutant) restored the WT Fg binding level (Fig. 1A). Thus, M1 serves as the major Fg-binding protein in our MIT1 strain. Similarly, we asked whether the M1 protein is sufficient for Fg binding in heterologous expression studies in nonpathogenic *Lactococcus lactis* (Fig. 1B). No Fg binding was detected in *L. lactis* harboring an empty vector control in a plate-based assay. In contrast, Fg binding was markedly increased in *L. lactis* transformed with a plasmid expressing the WT M1 protein, but not M1 lacking its three main Fg-binding sites (M1 ΔFg) (26). Thus, as expressed on the surface of intact bacteria, the M1 protein is both necessary and sufficient for Fg binding.

M1 Protein Reduces Pharyngeal Cell Adherence and Invasion, with Binding to Fg Further Reducing Adherence—To date, the role of Fg binding by the M protein in GAS virulence has been analyzed in models of systemic infection such as blood survival, resistance to phagocytic killing, and endothelial cell interactions (24–26, 34, 39, 40). Here, we examined the effect of the M1 protein and its Fg-binding property on MIT1 GAS adherence to and invasion of pharyngeal epithelial cells, a critical first step in the pathogenesis of localized and systemic infections. For these studies, we analyzed the immortalized pharyngeal keratinocyte cell line OKP7/tert (hereafter referred to as OKP7), as previous work employed the Hep-2 line now recognized to be HeLa cells by karyotypic analysis (41). Interestingly,

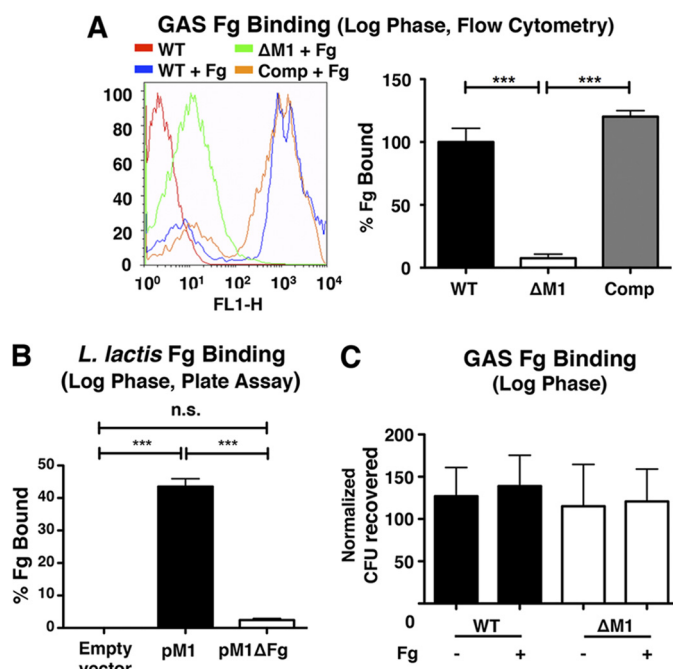


FIGURE 1. M1 protein is the major Fg-binding factor of MIT1 GAS strain 5448. A, flow cytometric analysis of WT and ΔM1 mutant GAS with FITC-labeled Fg: WT (red), WT + FITC-Fg (blue), ΔM1 + FITC-Fg (green), and complemented mutant (Comp) + FITC-Fg (orange). The bar graph shows quantification of the flow cytometry results. B, quantification of Fg binding by *L. lactis* strains expressing WT and Fg binding-deficient mutant versions of the M protein, expressed as a percentage of the original inoculum recovered bound to an Fg-coated plate. C, quantification of the GAS inoculum after binding to 8 $\mu\text{g}/\text{ml}$ Fg. Assays were performed in triplicate and repeated three independent times. ***, $p < 0.005$ (one-way analysis of variance). n.s., not significant.

we found that the ΔM1 mutant showed increased pharyngeal epithelial cell adherence (Fig. 2A) and invasion (Fig. 2B) compared with the WT parent GAS strain. This finding was consistent with our analysis of the adherence to and invasion of HaCaT skin keratinocytes (Fig. 3, A and B) and previous findings in the HaCaT cell line (42).

Normal Fg levels in human serum average $\sim 3 \text{ mg}/\text{ml}$ (43, 44), and Fg is detected in human sputum (2–20 $\mu\text{g}/\text{ml}$) (45) and saliva (46) and is localized on human tonsils (47). Upon addition of Fg at a conservatively low level of 8 $\mu\text{g}/\text{ml}$ (1/750th of normal serum levels), we observed a further reduction in OKP7 cell adherence by the WT and complemented mutant GAS strains, but not the ΔM1 mutant (Fig. 2A), consistent with our finding that the ΔM1 strain does not bind Fg (Fig. 1A). Although M1 inhibited intracellular invasion of OKP7 cells, the addition of Fg did not alter the level of inhibition (Fig. 2B). To verify that the observed effect of Fg was not due to GAS clumping resulting in reduced cfu, we incubated WT and ΔM1 GAS with and without 8 $\mu\text{g}/\text{ml}$ Fg and found no effect on GAS cfu recovery (Fig. 1C). We conclude that the M1 protein (and in particular, its ability to bind Fg) limits GAS adherence to pharyngeal keratinocytes *in vitro*. These findings were corroborated through direct imaging of FITC-labeled GAS bound to OKP7 monolayers (Fig. 2C).

To further explore the effect of M1 protein-Fg binding on GAS pharyngeal cell adherence, we examined the *L. lactis* strains expressing M1 and its Fg binding-deficient variant (M1 ΔFg). In the non-virulent, non-adherent *L. lactis* back-

Fg-binding M1 Protein Reduces GAS Pharyngeal Colonization

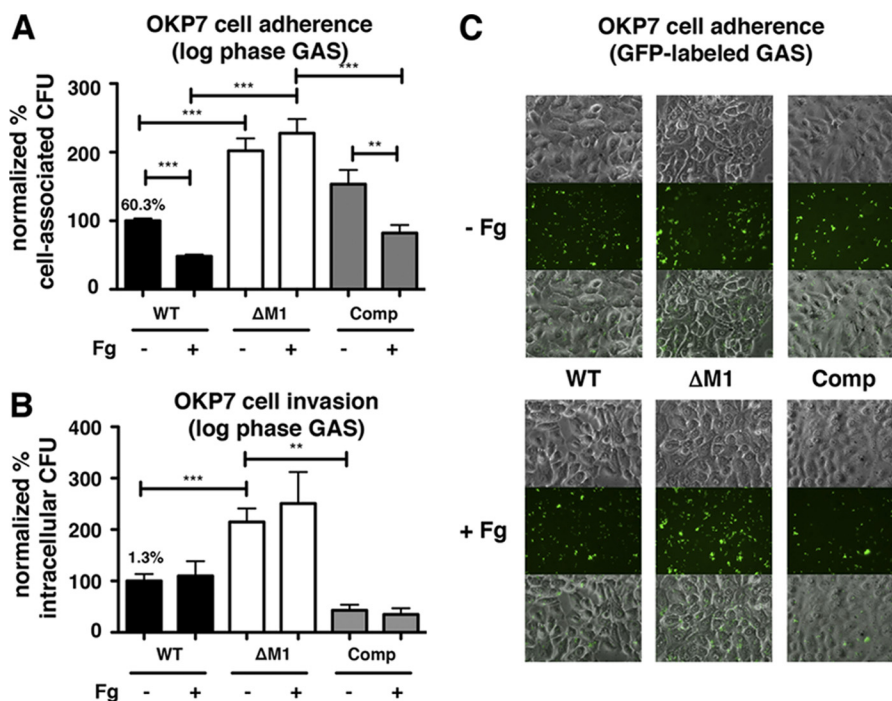


FIGURE 2. **M1 protein-Fg binding reduces adherence to pharyngeal keratinocytes.** Shown are the adherence to (A) and invasion of (B) OKP7 pharyngeal keratinocytes by logarithmic phase WT and Δ M1 mutant GAS in the presence or absence of 8 μ g/ml Fg, normalized to the inoculum. C, bright-field and fluorescence microscopy of FITC-labeled GAS adherence to OKP7 cells. FITC channel images were merged with bright-field images in Photoshop. Adherence/invasion assays were done in triplicate using triplicate sample wells. **, $p < 0.01$; ***, $p < 0.005$ (Student's t test). Comp, complemented mutant.

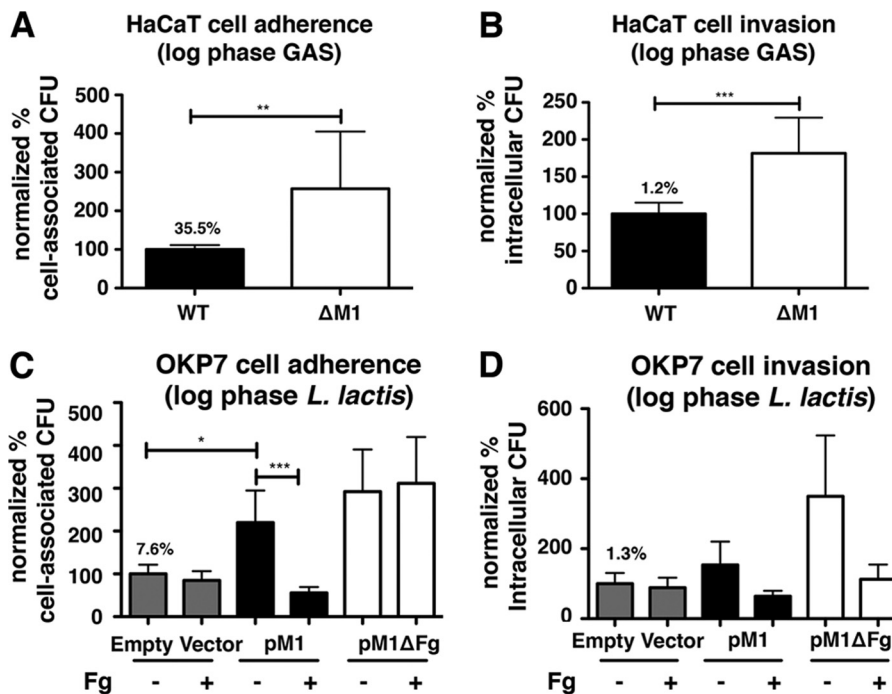


FIGURE 3. **M1 protein is sufficient to promote bacterial Fg binding and pharyngeal cell adherence, but differential effects are absent in stationary phase GAS.** Shown are the adherence to (A) and invasion of (B) HaCaT skin keratinocytes by logarithmic phase WT and Δ M1 mutant GAS. Also shown are the adherence to (C) and invasion of (D) OKP7 pharyngeal epithelial cells by logarithmic phase *L. lactis* expressing M1 protein constructs in the presence or absence of 8 μ g/ml Fg, normalized to the inoculum. Adherence/invasion assays were performed in triplicate and repeated independently three times. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Student's t test). ns, not significant.

ground, expression of the M1 protein increased adherence, although not to the level observed with GAS (7.6% versus 60.3% of inoculum) (Figs. 2A and 3C), indicating that the M1 protein can promote adherence to pharyngeal cells in the absence of other GAS adherence factors. This increase in adherence was

markedly diminished when Fg was added (Fig. 3C). Heterologous expression of M1 Δ Fg in *L. lactis* increased OKP7 cell adherence in a fashion that was not inhibited by Fg, showing that the Fg effect on adherence was mediated by the M1 protein (Fig. 3C). We did not observe any significant effects on *L. lactis*

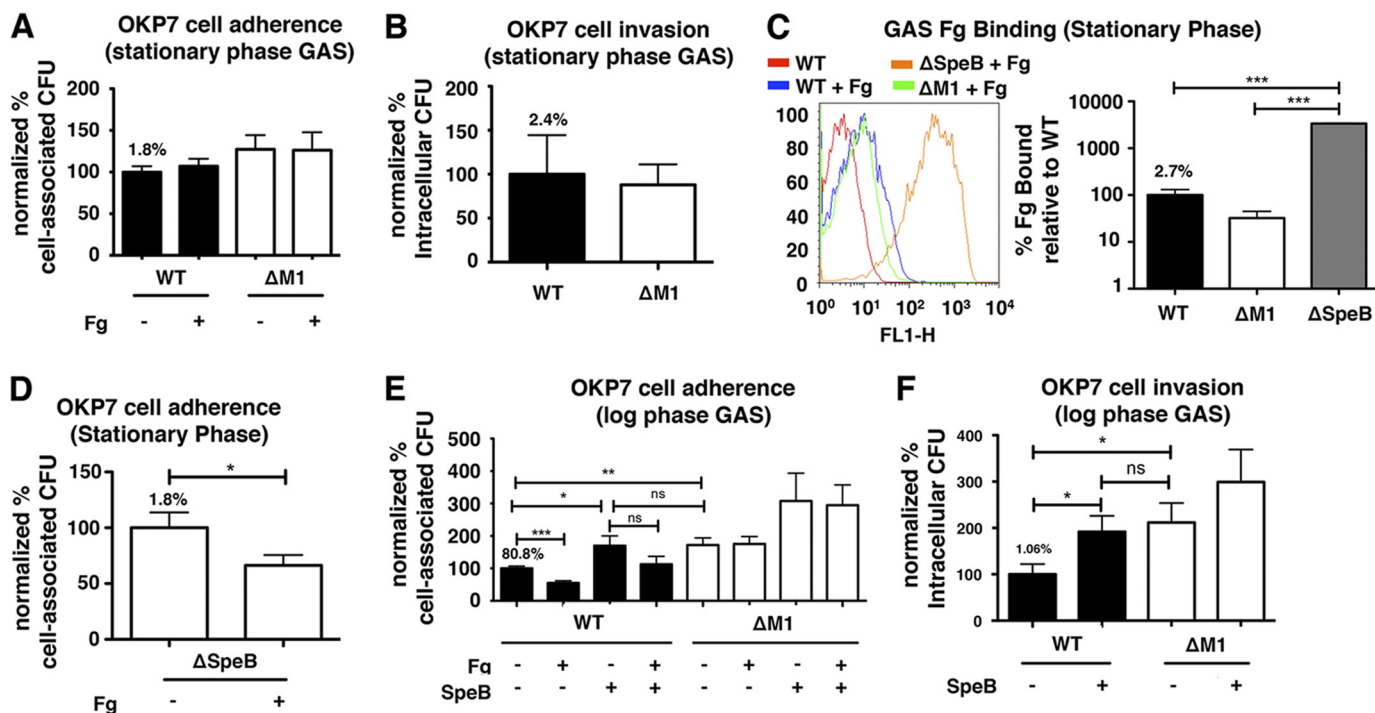


FIGURE 4. SpeB cleavage of the M1 protein counteracts M1- and Fg-mediated reductions in pharyngeal epithelial cell adherence. Shown are the adherence to (A) and invasion of (B) OKP7 pharyngeal keratinocytes by stationary phase WT and ΔM1 mutant GAS in the presence or absence of 8 μg/ml Fg (adherence only), normalized to the inoculum. C, left, flow cytometric analysis of stationary phase M1T1 GAS 5448 strains with FITC-labeled Fg: WT (red), WT + FITC-Fg (blue), ΔM1 + FITC-Fg (green), and ΔSpeB + FITC-Fg (orange). Right, quantification of flow cytometry with the GAS 5448 strains. D, adherence of stationary phase ΔSpeB mutant GAS to OKP7 pharyngeal epithelial cells in the presence or absence of 8 μg/ml Fg, normalized to the inoculum. Shown are the adherence to (E) and invasion of (F) logarithmic phase GAS to OKP7 cells in the presence or absence of 8 μg/ml Fg (adherence only) and 10 μg/ml recombinant SpeB, normalized to the inoculum. Fg binding assays were performed in triplicate and repeated three independent times. ***, $p < 0.005$ (one-way analysis of variance). Adherence and invasion assays were performed in triplicate and repeated three independent times. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ (Student's *t* test).

OKP7 cell invasion attributable to the M1 protein or Fg (Fig. 3D). We conclude that M1 protein binding to Fg reduces pharyngeal cell adherence in both native GAS and the *L. lactis* heterologous expression systems.

We next examined the growth phase dependence of M1 and Fg binding effects on pharyngeal cell adherence. Of note, we found that stationary phase WT and ΔM1 and stationary phase GAS had similar levels of OKP7 cell adherence and invasion in the presence and absence of Fg (Fig. 4, A and B), in contrast to the differences detected in log phase growth (Fig. 2, A and B). Thus, the differences between WT and ΔM1 detected in logarithmic phase were lost in stationary phase.

SpeB Proteolysis Reduces M1 Protein-dependent Effects on GAS Pharyngeal Cell Adherence—Expression of SpeB, a highly conserved broad-spectrum GAS protease (48) that cleaves M1 and other GAS target proteins, has been shown to influence surface expression of the GAS M1 protein in a growth phase-dependent manner. SpeB is expressed only in late logarithmic and stationary growth phase, leading to selective loss of surface M1 protein during stationary phase (20, 49, 50). We hypothesized that SpeB cleavage of surface M1 protein might account for the loss of Fg dependence on adhesion of M1T1 GAS. To understand the role of SpeB in GAS pharyngeal cell adherence, we examined ΔSpeB, a mutant generated in the same isogenic GAS background as ΔM1 (35) that allows the M1 protein to be preserved in all growth phases (20, 50). As predicted, elimination of SpeB to preserve M1 surface expression markedly

increased stationary phase Fg binding to the GAS surface compared with the negligible binding of Fg by the WT and ΔM1 strains (Fig. 4C). We also found that stationary phase adherence of the ΔSpeB mutant was susceptible to inhibition by Fg (Fig. 4D). These data support a model in which Fg bound to surface M1 protein limits epithelial cell adherence, which is counteracted in stationary phase by SpeB-mediated degradation of M1 (49, 50) and Fg itself (51). To more directly ascertain the effects of SpeB on adherence and invasion, we treated logarithmic phase cultures with recombinant SpeB protease. We observed an increase in the adherence and invasion of SpeB-treated logarithmic phase WT GAS relative to the untreated control (Fig. 4, E and F). This increase in adherence and invasion matched levels seen in the ΔM1 mutant, suggesting that SpeB treatment is functionally equivalent to genetic ablation of M1 with regard to pharyngeal cell adherence and invasion phenotypes. Additionally, SpeB-treated WT GAS was not susceptible to Fg inhibition, similar to the ΔM1 mutant (Fig. 4E). Finally, we did not detect a significant difference when treating ΔM1 with SpeB, indicating that the major effect of SpeB on OKP7 cell adherence and invasion is mediated through cleavage of M1 protein (Fig. 4, E and F).

M1 Protein Reduces GAS Colonization in a Mouse Upper Respiratory Tract Challenge Model—Because our combined studies in the tissue culture model indicated that M1 protein binding to Fg limits GAS pharyngeal cell adherence, we hypothesized that this phenotype could influence the pathogenesis of

Fg-binding M1 Protein Reduces GAS Pharyngeal Colonization

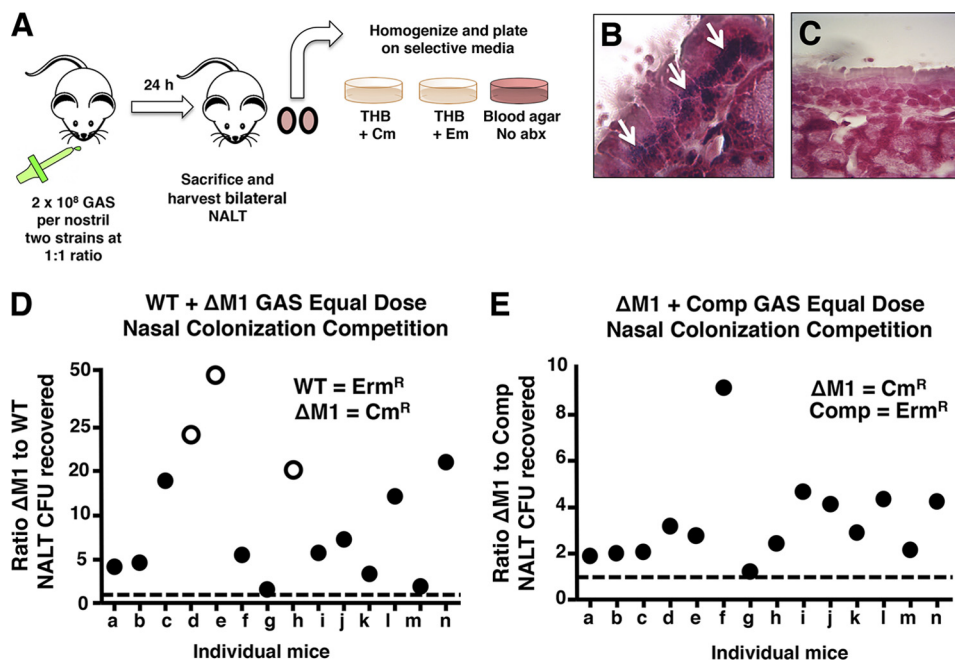


FIGURE 5. M1 protein reduces NALT colonization in a murine infection model. *A*, schematic of the infection model. Mice were nasally inoculated with a 1:1 mixture of two GAS strains, and NALT tissue was harvested at 24 h post-infection, homogenized, and plated on selective media. *Cm*, chloramphenicol; *Em*, erythromycin; *abx*, antibiotics. *B*, histological section of GAS-infected NALT tissue (Gram-stained). The *arrows* point to clusters of GAS. *C*, histological section of uninfected NALT tissue (Gram-stained). *D*, plot of the ratio of recovered Δ M1 GAS to WT GAS recovered. \circ , samples in which WT GAS was below detectable levels (limit of detection used for ratio calculation). The *dashed line* indicates a 1:1 ratio. *E*, plot of the ratio of recovered Δ M1 GAS to complemented mutant GAS. The *dashed line* indicates a 1:1 ratio.

GAS pharyngeal colonization/infection *in vivo*. Mouse NALT is thought to resemble and serve as a functional equivalent of human oropharyngeal lymphoid tissue (tonsils). It serves as a conduit to pulmonary tissues for respiratory pathogens and is the predominant source of GAS after nasal infection (38, 52). We utilized a competitive colonization assay to determine whether the Δ M1 GAS mutant displayed an *in vivo* advantage in NALT colonization as a result of increased adherence and invasion of pharyngeal keratinocytes. We chose a competitive model to overcome the differential effects between mice caused by the antigenic and proinflammatory properties of M proteins; in the competition model, WT and Δ M1 mutant bacteria are subject to the same magnitude and character of localized host response. C57BL/6 mice were infected intranasally with a 1:1 mixture of either (*a*) WT GAS *versus* Δ M1 mutant or (*b*) Δ M1 mutant *versus* complemented mutant (Fig. 5*A*). NALT was collected at 24 h post-infection, a time point at which histology confirmed the presence of abundant GAS within infected NALT (Fig. 5*B*) compared with the absence of bacteria in uninfected tissue (Fig. 5*C*). Differential plating was used to determine the number of viable GAS recovered for each strain (Fig. 5*A*). In the first competition assay, the ratio of Δ M1 mutant GAS to WT GAS recovered from 14 different mice ranged from 1.4:1 up to 48.8:1 (median = 6.4:1) (Fig. 5*D*). In the second competition assay, the ratio of Δ M1 mutant GAS to complemented mutant GAS recovered from 14 different mice ranged from 1.3:1 to 9.2:1 (median = 2.8:1) (Fig. 5*E*). These data clearly indicate a survival advantage of the Δ M1 mutant strain compared with the WT or complemented mutant strain in a short-term upper respiratory tract (NALT) colonization model, a finding that is compatible with Fg binding by the M1 protein limiting pharyngeal cell adherence.

DISCUSSION

In this work, we have shown that the M1 protein is the major Fg-binding protein for the globally disseminated M1T1 GAS clone and is sufficient to promote Fg binding when heterologously expressed in a nonpathogenic Gram-positive bacterium. Fg binding to GAS has been shown previously to protect against phagocytosis (22, 28, 39), and M1-Fg supramolecular networks activate neutrophils in a proinflammatory cascade that contributes to the pathogenesis of streptococcal toxic shock-like syndrome (25, 26, 40). These Fg-dependent properties establish the M1 protein as a virulence factor that promotes bacterial dissemination *in vivo* and fuels the systemic toxicity associated with severe invasive M1T1 GAS infection. In a different context, we found that Fg binding by the M1 protein limits GAS adherence to pharyngeal epithelial cells *in vitro* and NALT colonization *in vivo*. Establishment of GAS infection in the murine NALT model required a large bolus inoculum, which almost certainly triggers increased immune stimulation compared with the initial stages of human pharyngeal colonization. However, the data from this model were consistent with our adherence findings obtained in experiments using cultured human pharyngeal keratinocytes, which were performed at Fg concentrations found in the human pharyngeal microenvironment. Thus, we infer that our findings may be relevant to human pharyngeal colonization. The membrane cofactor protein CD46 has been reported to serve as a skin keratinocyte receptor for the conserved C repeat regions of the GAS M protein (53), and perhaps Fg binding by M1 interferes with this interaction. The markedly increased concentration of Fg in the blood *versus* the sputum (3 mg/ml *versus* 2 μ g/ml, respectively) indicates that the effects of M1-Fg binding to retard GAS phagocytosis or

promote inflammation are likely more intensive than its effects on pharyngeal colonization.

During the transition from localized to invasive M1T1 GAS infection, data from human isolates and mouse challenge studies indicate that mutants in the *covRS* two-component regulator arise under innate immune selection, altering the expression of multiple virulence factors and promoting a bloodstream-resistant phenotype (2, 18, 54, 55). Although M protein gene transcription itself is not significantly changed upon *covRS* mutation, two of the more striking changes in virulence phenotypes are up-regulation of the expression of the hyaluronic acid capsule and ablation of SpeB expression. Hyperencapsulation of the invasive *covRS* mutant promotes resistance to phagocytic clearance (56–58), yet the increase in hyaluronic acid coating has also been shown to limit M1 GAS epithelial cell adherence, biofilm formation, and *in vivo* colonization of skin (59).

We found that because SpeB cleaves the M protein from the GAS surface, it can mitigate the inhibitory effects of M1-mediated Fg binding on GAS pharyngeal epithelial cell binding, in particular during stationary phase when SpeB expression is maximal. Loss of SpeB in the invasive *covRS* mutant results in a *de facto* increase in surface M1 expression and Fg binding during bloodstream dissemination. Thus, intact *CovRS* regulation limits both capsule and surface M1 expression (via SpeB degradation) in the pharyngeal stage of infection, allowing maximal epithelial cell adherence. *CovRS* mutation *in vivo* causes hyperencapsulation and increased M1 protein surface expression, concurrently reducing pharyngeal binding while promoting phagocyte resistance and contributing mechanisms to the systemic dissemination and invasive infection for which the M1T1 clone is notorious. Our results provide further evidence of how bacterial virulence factors can exert differential effects on host cell interactions and disease pathogenesis based on the site and stage of infection.

REFERENCES

- Cunningham, M. W. (2000) Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **13**, 470–511
- Cole, J. N., Barnett, T. C., Nizet, V., and Walker, M. J. (2011) Molecular insight into invasive group A streptococcal disease. *Nat. Rev. Microbiol.* **9**, 724–736
- Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* **5**, 685–694
- Young, M. H., Aronoff, D. M., and Engleberg, N. C. (2005) Necrotizing fasciitis: pathogenesis and treatment. *Expert Rev. Anti-Infect. Ther.* **3**, 279–294
- Steer, A. C., Lamagni, T., Curtis, N., and Carapetis, J. R. (2012) Invasive group A streptococcal disease: epidemiology, pathogenesis and management. *Drugs* **72**, 1213–1227
- Cole, J. N., Henningham, A., Gillen, C. M., Ramachandran, V., and Walker, M. J. (2008) Human pathogenic streptococcal proteomics and vaccine development. *Proteomics Clin. Appl.* **2**, 387–410
- Steer, A. C., Batzloff, M. R., Mulholland, K., and Carapetis, J. R. (2009) Group A streptococcal vaccines: facts versus fantasy. *Curr. Opin. Infect. Dis.* **22**, 544–552
- Oehmcke, S., Shannon, O., Mörgelin, M., and Herwald, H. (2010) Streptococcal M proteins and their role as virulence determinants. *Clin. Chim. Acta* **411**, 1172–1180
- Metzgar, D., and Zampolli, A. (2011) The M protein of group A *Streptococcus* is a key virulence factor and a clinically relevant strain identification marker. *Virulence* **2**, 402–412
- Fischetti, V. A. (1989) Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* **2**, 285–314
- Fischetti, V. A. (1991) Streptococcal M protein. *Sci. Am.* **264**, 58–65
- McNamara, C., Zinkernagel, A. S., Macheboeuf, P., Cunningham, M. W., Nizet, V., and Ghosh, P. (2008) Coiled-coil irregularities and instabilities in group A *Streptococcus* M1 are required for virulence. *Science* **319**, 1405–1408
- Beall, B., Facklam, R., and Thompson, T. (1996) Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. *J. Clin. Microbiol.* **34**, 953–958
- Shulman, S. T., Tanz, R. R., Kabat, W., Kabat, K., Cederlund, E., Patel, D., Li, Z., Sakota, V., Dale, J. B., and Beall, B. (2004) Group A streptococcal pharyngitis serotype surveillance in North America, 2000–2002. *Clin. Infect. Dis.* **39**, 325–332
- Steer, A. C., Law, I., Matatolu, L., Beall, B. W., and Carapetis, J. R. (2009) Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development. *Lancet Infect. Dis.* **9**, 611–616
- Aziz, R. K., and Kotb, M. (2008) Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. *Emerg. Infect. Dis.* **14**, 1511–1517
- Tart, A. H., Walker, M. J., and Musser, J. M. (2007) New understanding of the group A *Streptococcus* pathogenesis cycle. *Trends Microbiol.* **15**, 318–325
- Sumby, P., Whitney, A. R., Graviss, E. A., DeLeo, F. R., and Musser, J. M. (2006) Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathog.* **2**, e5
- Kansal, R. G., McGeer, A., Low, D. E., Norrby-Teglund, A., and Kotb, M. (2000) Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. *Infect. Immun.* **68**, 6362–6369
- Aziz, R. K., Pabst, M. J., Jeng, A., Kansal, R., Low, D. E., Nizet, V., and 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, J. N., McArthur, J. D., McKay, F. C., Sanderson-Smith, M. L., Cork, A. J., Ranson, M., Rohde, M., Itzek, A., Sun, H., Ginsburg, D., Kotb, M., Nizet, V., Chhatwal, G. S., and Walker, M. J. (2006) Trigger for group A streptococcal M1T1 invasive disease. *FASEB J.* **20**, 1745–1747
- Carlsson, F., Sandin, C., and Lindahl, G. (2005) Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. *Mol. Microbiol.* **56**, 28–39
- McArthur, J. D., and Walker, M. J. (2006) Domains of group A streptococcal M protein that confer resistance to phagocytosis, opsonization and protection: implications for vaccine development. *Mol. Microbiol.* **59**, 1–4
- Sandin, C., Carlsson, F., and Lindahl, G. (2006) Binding of human plasma proteins to *Streptococcus pyogenes* M protein determines the location of opsonic and non-opsonic epitopes. *Mol. Microbiol.* **59**, 20–30
- Herwald, H., Cramer, H., Mörgelin, M., Russell, W., Sollenberg, U., Norrby-Teglund, A., Flodgaard, H., Lindbom, L., and Björck, L. (2004) M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* **116**, 367–379
- Macheboeuf, P., Buffalo, C., Fu, C. Y., Zinkernagel, A. S., Cole, J. N., Johnson, J. E., Nizet, V., and Ghosh, P. (2011) Streptococcal M1 protein constructs a pathological host fibrinogen network. *Nature* **472**, 64–68
- Courtney, H. S., Hasty, D. L., and Dale, J. B. (2002) Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. *Ann. Med.* **34**, 77–87
- Courtney, H. S., Hasty, D. L., and Dale, J. B. (2006) Anti-phagocytic mechanisms of *Streptococcus pyogenes*: binding of fibrinogen to M-related protein. *Mol. Microbiol.* **59**, 936–947
- Jenkins, H. T., Mark, L., Ball, G., Persson, J., Lindahl, G., Uhrin, D., Blom, A. M., and Barlow, P. N. (2006) Human C4b-binding protein, structural basis for interaction with streptococcal M protein, a major bacterial virulence factor. *J. Biol. Chem.* **281**, 3690–3697
- Ji, Y., Carlson, B., Kondagunta, A., and Cleary, P. P. (1997) Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization

Fg-binding M1 Protein Reduces GAS Pharyngeal Colonization

- of mice by the group A *Streptococcus*. *Infect. Immun.* **65**, 2080–2087
31. Wang, J. R., and Stinson, M. W. (1994) M protein mediates streptococcal adhesion to HEp-2 cells. *Infect. Immun.* **62**, 442–448
 32. Schrager, H. M., Alberti, S., Cywes, C., Dougherty, G. J., and Wessels, M. R. (1998) Hyaluronic acid capsule modulates M protein-mediated adherence and acts as a ligand for attachment of group A *Streptococcus* to CD44 on human keratinocytes. *J. Clin. Invest.* **101**, 1708–1716
 33. Chatellier, S., Ihendyane, N., Kansal, R. G., Khambaty, F., Basma, H., Norrby-Teglund, A., Low, D. E., McGeer, A., and 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
 34. Lauth, X., von Köckritz-Blickwede, M., McNamara, C. W., Myskowski, S., Zinkernagel, A. S., Beall, B., Ghosh, P., Gallo, R. L., and Nizet, V. (2009) M1 protein allows group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. *J. Innate Immun.* **1**, 202–214
 35. Kansal, R. G., Nizet, V., Jeng, A., Chuang, W. J., and Kotb, M. (2003) Selective modulation of superantigen-induced responses by streptococcal cysteine protease. *J. Infect. Dis.* **187**, 398–407
 36. Timmer, A. M., Kristian, S. A., Datta, V., Jeng, A., Gillen, C. M., Walker, M. J., Beall, B., and Nizet, V. (2006) Serum opacity factor promotes group A streptococcal epithelial cell invasion and virulence. *Mol. Microbiol.* **62**, 15–25
 37. Dickson, M. A., Hahn, W. C., Ino, Y., Ronfard, V., Wu, J. Y., Weinberg, R. A., Louis, D. N., Li, F. P., and Rheinwald, J. G. (2000) Human keratinocytes that express hTERT and also bypass a p16^{INK4a}-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics. *Mol. Cell. Biol.* **20**, 1436–1447
 38. Park, H. S., Francis, K. P., Yu, J., and Cleary, P. P. (2003) Membranous cells in nasal-associated lymphoid tissue: a portal of entry for the respiratory mucosal pathogen group A streptococcus. *J. Immunol.* **171**, 2532–2537
 39. Ringdahl, U., Svensson, H. G., Kotarsky, H., Gustafsson, M., Weineisen, M., and Sjöbring, U. (2000) A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance. *Mol. Microbiol.* **37**, 1318–1326
 40. Uchiyama, S., Andreoni, F., Zürcher, C., Schilcher, K., Ender, M., Madon, J., Matt, U., Ghosh, P., Nizet, V., Schuepbach, R. A., and Zinkernagel, A. S. (2013) Coiled-coil irregularities of the M1 protein structure promote M1-fibrinogen interaction and influence group A *Streptococcus* host cell interactions and virulence. *J. Mol. Med.* **91**, 861–869
 41. Chen, T. R. (1988) Re-evaluation of HeLa, HeLa S3, and HEp-2 karyotypes. *Cytogenet. Cell Genet.* **48**, 19–24
 42. Abbot, E. L., Smith, W. D., Siou, G. P., Chiriboga, C., Smith, R. J., Wilson, J. A., Hirst, B. H., and Kehoe, M. A. (2007) Pili mediate specific adhesion of *Streptococcus pyogenes* to human tonsil and skin. *Cell. Microbiol.* **9**, 1822–1833
 43. Ogston, C. M., and Ogston, D. (1966) Plasma fibrinogen and plasminogen levels in health and in ischaemic heart disease. *J. Clin. Pathol.* **19**, 352–356
 44. Poon, K. W., Lyng, F. M., Knief, P., Howe, O., Meade, A. D., Curtin, J. F., Byrne, H. J., and Vaughan, J. (2012) Quantitative reagent-free detection of fibrinogen levels in human blood plasma using Raman spectroscopy. *Analyst* **137**, 1807–1814
 45. Yoshinaga, S., Nakahori, Y., and Yasuoka, S. (1998) Fibrinolytic activity of a novel trypsin-like enzyme found in human airway. *J. Med. Invest.* **45**, 77–86
 46. Sukharev, A. E., Nikolaev, A. A., Nikolaeva, N. N., and Sukhareva, N. A. (1991) Lactoferrin and fibrinogen degradation products in saliva. *Lab. Delo.* **20**–24
 47. Lefevre, E. A., Hein, W. R., Stamatakis, Z., Brackenbury, L. S., Supple, E. A., Hunt, L. G., Monaghan, P., Borhis, G., Richard, Y., and Charleston, B. (2007) Fibrinogen is localized on dark zone follicular dendritic cells *in vivo* and enhances the proliferation and survival of a centroblastic cell line *in vitro*. *J. Leukoc. Biol.* **82**, 666–677
 48. Kapur, V., Topouzis, S., Majesky, M. W., Li, L. L., Hamrick, M. R., Hamill, R. J., Patti, J. M., and Musser, J. M. (1993) A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb. Pathog.* **15**, 327–346
 49. Berge, A., and Björck, L. (1995) Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.* **270**, 9862–9867
 50. Raeder, R., Woischnik, M., Podbielski, A., and Boyle, M. D. (1998) A secreted streptococcal cysteine protease can cleave a surface-expressed M1 protein and alter the immunoglobulin binding properties. *Res. Microbiol.* **149**, 539–548
 51. Matsuka, Y. V., Pillai, S., Gubba, S., Musser, J. M., and Olmsted, S. B. (1999) Fibrinogen cleavage by the *Streptococcus pyogenes* extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity. *Infect. Immun.* **67**, 4326–4333
 52. Hyland, K. A., Brennan, R., Olmsted, S. B., Rojas, E., Murphy, E., Wang, B., and Cleary, P. P. (2009) The early interferon response of nasal-associated lymphoid tissue to *Streptococcus pyogenes* infection. *FEMS Immunol. Med. Microbiol.* **55**, 422–431
 53. Okada, N., Liszewski, M. K., Atkinson, J. P., and Caparon, M. (1995) Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2489–2493
 54. Engleberg, N. C., Heath, A., Miller, A., Rivera, C., and DiRita, V. J. (2001) Spontaneous mutations in the CsrRS two-component regulatory system of *Streptococcus pyogenes* result in enhanced virulence in a murine model of skin and soft tissue infection. *J. Infect. Dis.* **183**, 1043–1054
 55. Walker, M. J., Hollands, A., Sanderson-Smith, M. L., Cole, J. N., Kirk, J. K., Henningham, A., McArthur, J. D., Dinkla, K., Aziz, R. K., Kansal, R. G., Simpson, A. J., Buchanan, J. T., Chhatwal, G. S., Kotb, M., and Nizet, V. (2007) DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nat. Med.* **13**, 981–985
 56. Wessels, M. R., and Bronze, M. S. (1994) Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12238–12242
 57. Dale, J. B., Washburn, R. G., Marques, M. B., and Wessels, M. R. (1996) Hyaluronate capsule and surface M protein in resistance to opsonization of group A streptococci. *Infect. Immun.* **64**, 1495–1501
 58. Moses, A. E., Wessels, M. R., Zalzman, K., Alberti, S., Natanson-Yaron, S., Menes, T., and Hanski, E. (1997) Relative contributions of hyaluronic acid capsule and M protein to virulence in a mucoid strain of the group A *Streptococcus*. *Infect. Immun.* **65**, 64–71
 59. Hollands, A., Pence, M. A., Timmer, A. M., Osvath, S. R., Turnbull, L., Whitchurch, C. B., Walker, M. J., and Nizet, V. (2010) Genetic switch to hypervirulence reduces colonization phenotypes of the globally disseminated group A streptococcus M1T1 clone. *J. Infect. Dis.* **202**, 11–19