Binding host proteins to the M protein contributes to the mortality associated with influenza–*Streptococcus pyogenes* superinfections

Andrea L. Herrera, Kuta Suso, Stephanie Allison, Abby Simon, Evelyn Schlenker, Victor C. Huber and Michael S. Chaussee*

**Abstract**

The mortality associated with influenza A virus (IAV) is often due to the development of secondary bacterial infections known as superinfections. The group A streptococcus (GAS) is a relatively uncommon cause of IAV superinfections, but the mortality of these infections is high. We used a murine model to determine whether the surface-localized GAS M protein contributes to the outcome of IAV–GAS superinfections. A comparison between wild-type GAS and an M protein mutant strain (emm3) showed that the M3 protein was essential to virulence. To determine whether the binding, or recruitment, of host proteins to the bacterial surface contributed to virulence, GAS was suspended with BALF collected from mice that had recovered from a sub-lethal infection with IAV. Following intranasal inoculation of naïve mice, the mortality associated with the wild-type strain, but not the emm3 mutant strain, was greater compared to mice inoculated with GAS suspended with either BALF from uninfected mice or PBS. Further analyses showed that both albumin and fibrinogen (Fg) were more abundant in the respiratory tract 8 days after IAV infection, that M3 bound both proteins to the bacterial surface, and that suspension of GAS with either protein increased GAS virulence in the absence of antecedent IAV infection. Overall, the results showed that M3 is essential to the virulence of GAS in an IAV superinfection and suggested that increased abundance of albumin and Fg in the respiratory tract following IAV infection enhanced host susceptibility to secondary GAS infection.

**INTRODUCTION**

Influenza A virus (IAV) causes respiratory illness, which is associated with relatively low mortality among healthy adults; however, secondary bacterial infections, or superinfections, greatly increase the morbidity and mortality associated with influenza during both seasonal influenza epidemics and pandemics [1–6]. *Streptococcus pneumoniae* is responsible for most secondary infections, while cases involving *Streptococcus pyogenes* [the group A streptococcus (GAS)] occur less frequently. Nonetheless, IAV–GAS superinfections are associated with high mortality, even when patients are treated with antibiotics that are effective against GAS *ex vivo* [7]. During the 2009 H1N1 IAV pandemic, GAS was responsible for nearly one-third of all deaths attributed to IAV superinfection [8]. Moreover, epidemiological studies suggest that influenza infections contribute to the development of invasive GAS (iGAS) diseases other than pneumonia, including bacteremia, toxic shock syndrome and necrotizing fasciitis [7]. Compared to superinfections caused by *S. pneumoniae*, less is known about the pathogenesis of IAV–GAS superinfections.

IAV infection alters the environment of the respiratory tract. There are changes in the cellular composition due to an influx of immune effector cells and viral-induced lysis of host epithelial cells [9]. In addition, patients with severe infection typically suffer from acute lung injury resulting in increased pulmonary microvascular leakage [10]. This is also associated with an increase in plasma levels of heparin-binding protein (HBP; also called azurocidin or CAP37), a granule-associated protein secreted by polymorphonuclear leukocytes (PMNs), and an increase in the risk of bacteremia [11–14]. Furthermore, IAV infection elicits a...
thrombotic inflammatory response, which increases the abundance of fibrinogen (Fg)/fibrin and fibronectin (Fn) in the lung [15].

The GAS M protein, encoded by the emm gene, is localized to the bacterial cell wall and binds to a variety of host proteins, which influences interactions with the host [16]. There are more than 200 M-protein serotypes and type-specific antibodies confer protection against infection by that specific serotype [16]. The M protein inhibits phagocytosis and is often considered to be the single most important virulence factor of GAS [17]. Host proteins known to bind to the M protein include Fg [18], albumin [19–21], C4b-binding protein (C4BP) [22] and factor H [23]. Fg binds to several M serotypes, including serotype M3 [24], and Fg binding to the M protein is essential for M protein-dependent inhibition of phagocytosis [25, 26]. Albumin binds to the C-repeats (present in the carboxyl region of the protein) of the M protein and inhibits antibody opsonization to this region of the protein [27]; however, the significance of albumin binding to the M protein in a naive host is not clear. The binding of C4BP and factor H to the surface of GAS decreases complement opsonization [28]. In general, the contribution of the M protein to the virulence of GAS is largely dependent on the binding, or recruitment, of host proteins to the bacterial surface.

GAS serotype M3 (as well as M1 and M89) is commonly associated with IAV superinfections [29], pneumonia and other iGAS diseases [30]. One clinical isolate, designated MGAS315, is genetically representative of contemporary M3 strains [31]. The M3 protein is required for virulence in a GAS mono-infection of mice [32] and antibodies against the protein are protective [33]. In addition, we previously showed that vaccination of mice against the M3 protein conferred complete protection against IAV–GAS superinfection [34].

The purpose of this study was to determine whether the M protein is important to GAS virulence in the context of an IAV superinfection. The results obtained with a murine model of IAV–GAS superinfection showed that the M protein is required for virulence in an IAV–GAS superinfection. The results also suggested that changes in the abundance and composition of host proteins in the respiratory tract in response to IAV infection contributed to the increased susceptibility to secondary GAS infection.

METHODS

Strains and culture conditions

S. pyogenes strain MGAS315 (serotype M3) was obtained from ATCC and grown without agitation with Todd–Hewitt broth (BD Biosciences, San Jose, CA, USA) supplemented with 0.2 % yeast extract (THY) at 37 °C in 5 % CO₂ until the mid-exponential phase of growth. MGAS315 has a growth yield of approximately 1.0 absorbance unit at 600 nm (A₆₀₀) [35]. The mid-exponential phase was defined as a culture with an A₆₀₀ of approximately 0.5, which was attained by both the wild-type and the emm3 mutant strain after approximately 2.5 to 3.5 h of incubation. GAS was also grown with THY agar and triple agar containing 5 % sheep blood. When appropriate, the emm3 mutant strain (emm3) was grown with THY containing 2.5 µg ml⁻¹ of erythromycin. Escherichia coli strain DH5α was purchased from Gibco-BRL (Gaithersburg, MD, USA) and grown with Luria–Bertani medium. When appropriate erythromycin was added at 200 µg ml⁻¹.

Directed mutagenesis of the emm3 gene

A portion of the emm3 open reading frame (ORF) was amplified using the primers (5′-GCTTGAAATTCGAC TTGCCCAAACGGGTATA-3′) and (5′-GTCTTAAAGC TTTGTGGTACCTAATGCTGCT-3′), and the ampli- con was digested with EcoRI and HindIII. The resulting 376 bp fragment was cloned into the suicide vector pVA891-2, which replicates in E. coli but not in S. pyogenes [36]. Following E. coli transformation, the recombinant plasmid was isolated and used to transform GAS, essentially as previously described [37]. Briefly, S. pyogenes MGAS315 was inoculated from agar plates into 40 ml of THY in 50 ml Corning tubes and incubated overnight at 37 °C in 5 % CO₂ without agitation. The bacteria were collected by centrifugation and washed four times with 20 ml of 15 % glycerol prior to being suspended with 300 µl of 15 % glycerol. One hundred µl aliquots of washed cells were then stored at −80 °C prior to electroporation. For electroporation, frozen aliquots of MGAS315 were thawed on ice and 5 µl of 3 µg µl⁻¹ transforming DNA was added. As a negative control, 5 µl of water was added to a separate aliquot of cells. The mixtures were each added to separate Gene Pulser cuvettes (Biorad; 2 mm gap) and electroporated with a BioRad Gen Pulse Excell instrument with the following settings: capacitance, 25 µF; resistance, 200 ohms; volts, 2.1 kV. The bacteria were suspended in 10 ml of THY and incubated for 2 h at 37 °C with 5 % CO₂ prior to plating on THY agar containing 2.5 µg ml⁻¹ erythromycin. The plates were incubated at 37 °C with 5 % CO₂. Inactivation of the gene was confirmed by PCR and nucleotide sequencing. The heterologous DNA was inserted into the emm3 coding sequence at the 5′ region of the open reading frame 467 base pairs from the initiation codon.

Murine model of infection

The University of South Dakota is a licensed research facility under the authority of the United States Department of Agriculture and the facility registration number is USDA License 46 R-001. All of the animal experiments described in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of South Dakota (protocol number 11-07-15-18E). Adult (6 to 8-week-old) female BALB/cj mice were obtained from Harlan Laboratories (Indianapolis, IN, USA) and housed in groups of four with 24 h access to food and water. The
Host protein binding to the surface of MGAS315

The wild-type MGAS135 strain and emm3 mutant were grown to the mid-exponential phase of growth, washed two times with sterile phosphate-buffered saline (PBS; pH 7.4) and suspended with PBS. Subsequently, 10^6 c.f.u. were diluted in 100 µl PBS and incubated with either 5 µg ml⁻¹ purified Fg (Oxford Biomedical Research; Oxford, MI, USA), albumin (Thermo/Fisher) or 100 µl bronchoalveolar lavage fluid (BALF) at room temperature for 10 min, and kept on ice until intranasal inoculation.

Viral and bacterial titres

To measure the dissemination of IAV and GAS, groups of mice were euthanized 24 h after infection with MGAS315 (8 days after HK68 virus infection) and the lungs, spleen and blood were collected as described previously [38]. Briefly, lung homogenates were serially diluted and plated onto Todd–Hewitt agar plates, agar plates containing sheep blood, or Madin–Darby canine kidney (MDCK) monolayers to determine the bacterial (c.f.u. ml⁻¹) and viral titres (TCID₅₀), respectively. Spleen homogenates were serially diluted and plated onto Todd–Hewitt agar or agar plates containing sheep blood to enumerate the CFUs. Fluids and tissue samples from mice infected with the emm3 mutant strain were also simultaneously plated on agar plates that contained 2.5 µg ml⁻¹ of erythromycin and on plates that did not contain erythromycin to determine whether the chromosomal insertion conferring resistance to erythromycin was unexpectedly lost during murine infection.

Bacterial titre determination

The bacterial titre was determined using a C6 flow cytometer (Accuri Cytometers, Ltd, Ann Arbor, MI, USA) using CFlow Plus software (Accuri).

BALF collection and cytokine analyses

Mice were inoculated with a sub-lethal dose of IAV and after 7 days a sub-lethal dose of GAS or sterile PBS, as previously described. At indicated time points, the mice were euthanized and BALF collected using 1 ml of sterile PBS containing 5 mM EDTA per mouse. The BAL fluids were centrifuged at 5000 g for 10 min and the supernatants were stored at –80 °C.

Selected cytokines in BALF were measured using a cytometric bead array kit (BD Biosciences, San Diego, CA, USA), following the manufacturer’s instructions. Samples were analysed with a C6 flow cytometer (Accuri Cytometers, Ltd, Ann Arbor, MI, USA) using CFlow Plus software (Accuri).

Lung collection, fixation and staining

Eight days after IAV infection, the mice were euthanized and the lungs removed and fixed in 4 % paraformaldehyde. The left lung lobe of each mouse was subsequently dehydrated in a series of alcohol solutions followed by submersion in a clearing agent (Fisherbrand CitriSolv) for 1 h. The samples were transferred through three changes of melted paraffin, embedded into wax blocks, sliced longitudinally and affixed to slides. Haematoxylin and eosin (H&E) staining was used to visualize the lung structure and analyse the progression of infection and inflammation.

For immunohistochemistry (IHC), paraffin-embedded lung slices underwent an antigen retrieval protocol in which slices were placed into a pre-heated sodium citrate buffer solution for 10 min. The slides were then washed in 0.1 M PBS and incubated in a PBS H₂O₂ solution for 30 min, washed with 0.1 M PBS and incubated in a goat blocking buffer for 1 h. The primary antibodies – anti-Fg (Abcam; 1 : 1000) or anti-GAS (Thermo Scientific PA1-73059; 1 : 5000) – were incubated with the samples for 24 h. The next day, the samples were washed three times with 0.1 M PBS and incubated for 1 h in secondary goat-anti-rabbit antibody (Vector Laboratories, CA, USA; 1 : 2000). After the slides were washed again in PBS, they were incubated in AB enzyme reagent for 1 h, washed in PBS and then incubated in 3,3’-diaminobenzidine solution (DAB) for 5 to 10 min. The DAB solution was removed with another PBS wash. The slides were counterstained with haematoxylin and cleared in tap water. The samples were then dehydrated in a series of increasing alcohol grades followed by CitriSolv, as previously described.

Image analysis

The histopathology and IHC scoring for the lung slices were conducted in a double-blind manner. The pathology was scored according to the thickness and frequency of the collars surrounding most airways: 0, no pathology; 1, small collar (one layer of lymphocytes), 2, collar (two–four cells...
RESULTS

Inactivation of emm3 abolished virulence in both a GAS monoinfection and an IAV–GAS superinfection

To measure the contribution of the M protein to the morbidity and mortality associated with IAV–GAS superinfections, the gene was inactivated in the serotype M3 strain MGAS315. There was no difference between the parental and mutant strains in growth rate or growth yield when cultured with THY broth, or haemolytic activity when cultured with agar plates containing sheep blood (data not shown). The virulence of the mutant strain was assessed in a monoinfection of mice. The LD_{50} [40] of the parental MGAS315 strain was \(10^{7.5} \text{cfu} \cdot \text{ml}^{-1}\) following i.n. inoculation of BALB/cJ mice, while that of the emm3 mutant strain was \(10^{6.5} \text{cfu} \cdot \text{ml}^{-1}\). The results showed that M3 contributed to the virulence of GAS when mice were intranasally infected with strain MGAS315.

To determine whether the emm3 gene was also important to virulence in an IAV–GAS superinfection, groups of mice were inoculated with a sub-lethal dose of IAV followed 7 days later by a sub-lethal dose of either wild-type MGAS315 or the emm3 mutant. All of the mice lost weight during the first 5 days of viral infection (Fig. 1a). Beginning on day 6, mice started to gain weight, indicating that they had recovered from the viral infection (Fig. 1a), and on day 7 the mice were inoculated with a sub-lethal dose of GAS that is not lethal in a monoinfection; however, when preceded by IAV infection, 59% of the mice infected with wild-type GAS strain reached end-point criteria (Fig. 1b). In contrast, none of the mice inoculated with the emm3 mutant reached end-point criteria (P<0.05; Fig. 1b). The results showed that the M3 protein is required for GAS virulence in a murine model of IAV–GAS superinfection.

Inactivation of emm3 reduced GAS survival in the lung

Lungs, BALF, spleens and blood were collected from superinfected mice 24h after inoculation with GAS (8 days after inoculation with virus) and the viral and bacterial titres were measured. IAV was recovered from the lungs of 1 mouse out of 11, indicating that most mice had cleared the virus from the lower respiratory tract 8 days after inoculation (data not shown). The absence of viral infection in most mice after 8 days of IAV infection was consistent with the observation that mice began to gain weight 5 days after IAV inoculation, and with our previous findings [38]. Among mice inoculated with IAV (day 0) and wild-type MGAS315 (day 7), an average of \(10^{1.5} \text{cfu} \cdot \text{ml}^{-1}\) of GAS were recovered from the lungs 24h after bacterial infection (day 8; Fig. 2a). In contrast, \(10^{3.5} \text{cfu} \cdot \text{ml}^{-1}\) were recovered from mice superinfected with the emm3 mutant (Fig. 2a; P<0.05). Similarly mice infected with wild-type GAS had significantly more bacteria present in BALF compared to mice infected with the emm3 mutant (P<0.01; Fig. 2b).

To determine whether GAS disseminated from the lung to cause systemic infection, the number of viable bacteria in the spleen and blood of superinfected mice was measured. Mice inoculated with wild-type bacteria had an average of \(10^{3.5} \text{cfu} \cdot \text{ml}^{-1}\) in both the spleen and blood samples (data not shown). In contrast, mice inoculated with the emm3 mutant had an average of \(10^1\) and \(10^2 \text{cfu} \cdot \text{ml}^{-1}\) in the spleen and blood, respectively (data not shown). When tissue or fluids from mice inoculated with the emm3 mutant strain were plated on agar plates containing sheep red blood cells to enumerate the viable GAS, samples were simultaneously plated on media with and without erythromycin to determine whether the heterologous DNA encoding resistance to erythromycin was retained in the absence of selection in the mice. As expected, the results confirmed that the chromosomal insertion was stable in the mice (data not shown). Taken together, infection with IAV and wild-type MGAS315 resulted in a greater number of bacteria.
being present in the respiratory tract (lungs and BALF), blood and spleen 24 h after infection with GAS compared to the mutant strain. The persistence of infection, and increased dissemination of the wild-type strain, was associated with greater mortality compared to the mutant (Fig. 1b).

**Antecedent IAV infection increased pulmonary vascular barrier dysfunction, which was associated with GAS dissemination and mortality**

We hypothesized that an IAV–GAS superinfection might synergistically enhance barrier dysfunction due to both IAV and M protein-mediated activation of PMNs. To compare the barrier dysfunction among infected mice, the concentration of albumin in BALF was measured 24 h after superinfection with GAS (8 days after IAV infection) [41, 42]. Mice infected solely with IAV had higher albumin levels compared to uninfected controls (Fig. 3), which suggested that viral infection increased damage to the pulmonary epithelium. Superinfection with wild-type GAS resulted in a significant increase in albumin levels (P<0.05) compared to uninfected mice, which was indicative of increased barrier dysfunction (Fig. 3). In contrast, inoculation with the emm3 mutant did not significantly increase the amount of albumin in BALF (P>0.05; Fig. 3). The results indicated that increased pulmonary vascular barrier dysfunction was associated with IAV superinfection with the wild-type strain, but not with the emm3 mutant, compared to uninfected controls; however, there was not a statistically significant difference between IAV infection alone and superinfection, or between superinfection with the wild-type and emm3 mutant strains.

**Inactivation of emm3 decreased the production of proinflammatory cytokines during IAV superinfection**

The concentrations of selected cytokines in BALF collected from superinfected mice (24 h after inoculation with GAS)
were determined. The concentrations of IL-2, IL-4, IL-17, IL-6 and TNFα were greater in mice superinfected with the wild-type strain compared to mice infected with the emm3 mutant strain or mice inoculated with virus and PBS as a control, although these trends were not statistically significant (P > 0.05; Fig. 4). IFNγ was significantly elevated in mice inoculated with the wild-type strain compared to those superinfected with the mutant strain (P < 0.05; Fig. 4). Although most of the differences in cytokine levels between mice superinfected with the wild-type or mutant strain were not statistically significant, IAV superinfection with the emm3 mutant was generally associated with decreased production of proinflammatory cytokines compared to superinfection with the parental strain.

**IAV–GAS superinfection increased the abundance of Fg in the respiratory tract**

The pathology of H&E-stained lung slices was scored for peribronchiolar, perivascular and parenchymal infiltrates. Infiltrates present in peribronchiolar connective tissue presented as a collar surrounding the airway, and in severe infections, formed a lymphoid mass.

---

**Fig. 3.** Inactivation of emm3 decreased the pulmonary microvascular dysfunction of superinfected mice. The amount of albumin in BALF samples independently isolated from groups of four mice each was determined as an indicator of pulmonary microvascular barrier dysfunction. The groups included uninfected control mice, mice infected only with IAV (IAV) and mice infected 7 days after IAV infection with either wild-type GAS (IAV+wild type) or the emm3 mutant (IAV+emm3). One-way ANOVA with Tukey’s post-test was used to analyse the data and the statistically significant (P<0.05) result is indicated by *; comparisons among other groups were not statistically significant (P>0.05).

**Fig. 4.** Inactivation of emm3 decreased the production of proinflammatory cytokines. Selected cytokines present in BALF, isolated from four individual mice for each group, were measured 8 days after infection with IAV (IAV), or 24 h after superinfection with wild-type MGAS315 (IAV+wild type) or the emm3 mutant strain (IAV+emm3). Non-parametric Kruskal–Wallis or Mann–Whitney analyses were used. Statistically significant differences are shown (P<0.05).
IAV infections are prothrombotic in humans, which can result in an increase in Fg at the site of infection [15, 43–45]. Because Fg is an important ligand of the M protein, it was of interest to measure the abundance of Fg in our murine model of IAV–GAS superinfection. The relative amounts of Fg present in BALF 24 h after GAS inoculation (8 days after IAV infection) were determined by immunoblotting. Superinfection with the wild-type strain was associated with increased Fg compared to mice superinfected with the emm3 mutant (Fig. 5). In addition, IHC was used to detect Fg in the lungs of mice and the results were consistent with those obtained by immunoblotting (data not shown). Representative supplementary figures are included to illustrate the scoring of inflammation (Fig. S1, available in the online Supplementary Material), the presence of Fg as revealed by IHC (Fig. S2) and examples of lung pathology (Fig. S3). Finally, the abundance of Fg in the lungs correlated positively with inflammation, as determined by IHC and H&E staining (P<0.01; Fig. 6).

**Identification of murine proteins that bind to the GAS surface**

Surface-localized proteins of GAS bind to a variety of host proteins, which contributes to both adherence and evasion of host effector cell clearance. The M3 protein binds to albumin and Fg, and binding of the latter confers resistance to phagocytosis [25, 26]. We hypothesized that the increased abundance of Fg in the respiratory tract following IAV infection (Figs 5 and 6) may contribute to the virulence of GAS in an IAV superinfection. Because GAS strains often express more than one surface protein that binds to the same host protein, it was necessary to determine the extent to which M3 in strain MGAS315 mediated binding to Fg. First, equal amounts (10^8 c.f.u.s) of the wild-type and *emm3* mutant strains were incubated with purified murine Fg and washed thoroughly, and then the Fg bound to the bacterial surface was detected by immunoblotting. Fg binding was decreased in the *emm3* mutant strain compared to the parental strain (P<0.01; Fig. 7). The results indicated that M3 was a Fg-binding surface protein, although there was also some Fg associated with the mutant strain (Fig. 7a, c). The results obtained by incubating the wild-type and mutant strains with murine plasma showed that in this context Fg binding was dependent on M3 (P<0.001; Fig. 7b, d).

IAV infections alter the abundance and composition of proteins within the lung, including cytokines. We noted that 8 days after IAV infection, albumin levels remained elevated (Fig. 3), even though at this time IAV was no longer detected in the lung. The results indicated that IAV alters the proteome of the lung, even after the virus is cleared. We were interested in determining whether IAV-induced changes in lung protein composition impacted on GAS recruitment of host proteins to the bacterial cell surface. To address this we examined the binding of murine proteins present in BALF obtained from mice infected with IAV, or not, to the GAS surface. Specifically, the wild-type and *emm3* mutant strains were incubated with BALF obtained from uninfected mice or mice inoculated 7 days prior with a sub-lethal dose of IAV. After incubation, the bacteria were washed thoroughly and surface-bound proteins were separated by SDS-PAGE (Fig. 8). Proteins of interest were excised from the gel and identified using
tandem mass spectrometry (data not shown). More murine albumin (and GAS enolase) was associated with the surface of wild-type GAS following incubation with BALF compared to the \textit{emm3} mutant (Fig. 8). In addition, more albumin was bound to the GAS surface following incubation with BALF from IAV infected mice compared to uninfected controls (Fig. 8). The results showed that M3 is mostly responsible for the binding of albumin to the bacterial cell surface.

**Suspension of GAS with BALF from IAV-infected mice or with purified albumin or Fg enhanced virulence in the absence of antecedent IAV infection**

We speculated that the increased abundance of albumin and Fg in the lower respiratory tract of mice after an IAV infection may enhance binding of these proteins to the GAS surface and promote bacterial persistence and subsequent dissemination. To address this possibility, wild-type MGAS315 or the \textit{emm3} mutant were suspended with BALF collected 7 days after the mice were inoculated with either a sub-lethal dose of IAV or allantoic fluid; naïve mice (mice not previously infected with IAV) were then inoculated intranasally. The morbidity (Fig. 9a) and mortality (Fig. 9b) of the mice were monitored for 13 days.

The mortality of mice inoculated with either wild-type GAS or the \textit{emm3} mutant suspended in BALF from uninfected mice was 20 and 15\%, respectively (\textit{P}>0.05; Fig. 9b).

![Fig. 7. Inactivation of \textit{emm3} decreased the binding of Fg to the bacterial surface. The wild-type and \textit{emm3} strains were incubated with (a) purified Fg or (b) murine plasma and washed thoroughly. Proteins associated with bacteria (bound) and those in the wash solution (unbound) were separated by SDS-PAGE and Fg was detected with Western blotting. Representative blot from four independent experiments using Fg and representative blots from four additional independent experiments using plasma are shown. Quantitative values were obtained by densitometry for strains incubated with Fg (c) or plasma (d) and the differences between wild-type and the \textit{emm3} mutant analysed with the Student \textit{t}-test; \textit{P} values <0.05 are designated with an *.

![Fig. 8. M3-bound albumin present in murine BALF. The wild-type (lanes 1 and 3) and \textit{emm3} mutant (lanes 2 and 4) were incubated with BALF obtained and pooled from four IAV-infected mice (lanes 1 and 2) or control mice inoculated with allantoic fluid (lanes 3 and 4). BALF was isolated from four mice and pooled. After washing, proteins were separated by electrophoresis and the bands indicated with letters (a–f) were excised and identified with mass spectrometry. Fg (lane 5) and albumin (Alb; lane 6) were loaded as references. A representative image is shown.](image-url)
To determine whether the recruitment of Fg or albumin to the bacterial surface contributed to the effect, wild-type MGAS315 and the emm3 mutant strain were suspended in PBS containing either purified murine albumin or Fg prior to intranasal inoculation of naïve mice. Suspension of wild-type GAS with either protein increased mortality (Fig. 9d, f) to a level similar to that observed when GAS was suspended in BALF obtained from mice not infected with IAV (Fig. 9b). The increase in virulence when suspended in Fg was dependent on the M3 protein (Fig. 9d), indicating it was likely due to M3 recruitment of Fg to the GAS surface. Infection with the emm3 mutant suspended in either Fg (P<0.001) or albumin (P<0.0001) resulted in reduced morbidity (Fig. 9c, e) and mortality (Fig. 9d, f) compared to the wild-type strain, although the difference in mortality was not statistically significant (P>0.05). Together, the results suggested that changes in the abundance and composition of host proteins, such as albumin and Fg, in the respiratory tract in response to IAV infection may contribute to host susceptibility to secondary GAS infection.

**DISCUSSION**

The host environment is significantly altered by viral infection. The response to IAV infection includes changes in cell
types, protein composition and abundance, including cytokines, and endothelial and effector cell function. These changes in the local environment are likely to impact on host-pathogen interactions, which could make classically defined virulence factors such as the M protein less important in this context. It was thus of interest to determine whether the M protein, which is a critical virulence factor in a GAS monoinfection, was similarly important for the outcome of IAV superinfections. We found that the M3 protein was essential for mortality in a murine model of IAV–GAS superinfection. In addition, we found that suspension of GAS with BALF obtained from mice that had recently recovered from an IAV infection and subsequent inoculation of naïve mice increased mortality, even in the absence of antecedent viral infection. Some of the effect was likely due to the presence of proinflammatory cytokines present in BALF; however, suspension of wild-type GAS, but not the emm3 mutant strain, with purified albumin or Fg, which were more abundant following IAV infection, was also associated with increased morbidity and mortality compared to controls. Overall the results indicated that increased abundance of albumin and Fg, together with increased expression of proinflammatory cytokines in response to IAV infection, may contribute to the susceptibility to secondary GAS infection, probably in part by host protein binding to the M protein.

The limitations of the study include cases in which differences between the wild-type and emm3 mutant strain were not statistically different and the lack of a complemented mutant strain. Because of the latter, we cannot rule out the possibility that secondary mutations contributed to the differences observed with the emm3 mutant. In this regard, complementation of the mutant strain using shuttle plasmids was complicated by the loss of the plasmids during murine infection.

Superinfection with the wild-type GAS strain, but not with the emm3 mutant or infection with IAV alone, resulted in a statistically significant increase in albumin, implying decreased pulmonary barrier function. The M protein is localized to the bacterial surface and covalently attached to peptidoglycan [16]; however, it also exists as a soluble, or freely diffusible, form. While the pathophysiology of streptococcal lung injury is complex, a previous report showed that the soluble form of M protein binds to Fg and activates neutrophils, which contributes to lung injury [46]. Thus the combined effect of an increase in damage to the lung epithelium in response to viral infection and superinfection with wild-type GAS, but not the emm3 mutant, resulted in significant pulmonary barrier dysfunction, which was associated with bacterial dissemination from the lung. In addition to M protein activation of neutrophils, it is also likely that the ability of the wild-type strain, but not the mutant, to sustain infection contributed to barrier dysfunction by prolonging the inflammatory response.

Previous results obtained with protein chips showed that M3 was the only surface-localized protein of strain MGAS315 to bind to Fg [47]. Our results support this finding, although some Fg was detected on the surface of the emm3 mutant strain when the mutant was incubated with purified Fg; however, surface-bound Fg was not observed when the mutant was incubated with plasma. It seems likely that surface proteins other than M3 bound purified Fg with low affinity in the absence of competing proteins; however, when incubated with plasma, the presence of other proteins such as albumin diminished binding.

The increased abundance of Fg in the respiratory tract of IAV-infected mice may increase host susceptibility to IAV–GAS secondary infection by at least four mechanisms. First, neutrophil activation by M3–Fg complexes can result in an increase in pulmonary barrier dysfunction and subsequent GAS dissemination from the lung, as discussed previously. Second, Fg bound to surface-localized M protein can inhibit the opsonization of GAS and killing by immune effector cells, thereby allowing the infection to persist. Third, Fg is a damage-associated molecular pattern protein [48] that can stimulate the expression of pro-inflammatory cytokines by binding to toll-like receptor 4 and further contribute to the severity of infection [49]. Finally, M3 bound to Fg mediates both adherence to epithelial cells and internalization, and the latter may sequester GAS from the immune response. To mediate adherence, Fg can act as a molecular bridge between host cell receptors, including integrins, and the M3 protein [50, 51]. However, studies with an M1 serotype strain showed that Fg decreased GAS adherence to keratinocytes [52]. Thus while further study is required to determine the precise mechanisms involved, the increase in mortality following the suspension of the wild-type strain, but not emm3 mutant, with purified Fg suggests that M3–Fg interactions are important.

The increased abundance of albumin in BALF after IAV infection may enhance its binding to the M3 protein on the surface of GAS during IAV–GAS superinfection. Albumin bound to the M protein decreases the efficacy of bacterial opsonization with M-type specific antibodies [27]; however, the contribution to virulence of albumin–M protein binding in an immunologically naive host is not clear. Our results showed that suspending GAS in albumin enhanced virulence compared to when bacteria were suspended in PBS. Moreover, the effect was more pronounced in the wild-type strain compared to the emm3 mutant strain. The results suggest that part of the decreased virulence associated with the emm3 mutant in IAV–GAS superinfections was due to an inability to recruit albumin to the bacterial surface.

Overall, the results of our study showed that the surface-localized M3 protein, which binds albumin and Fg to the bacterial surface, was essential to virulence in a murine model of IAV–GAS superinfection. Our results, and those of others [53], indicate that GAS virulence factors classically defined with monoinfection models are similarly critical in determining the outcome of IAV–GAS superinfections. Nonetheless, given the multi-factorial nature of iGAS disease and the increased susceptibility of the host to GAS
inflammation associated with pulmonary barrier dysfunction, resulting in the persistence of infection, prolonged inflammation associated with pulmonary barrier dysfunction and bacterial dissemination.

Funding information
Funding for the project was provided by NIH grant 1R15AI094437-01A1 from NIAID and P20 RR016479 from the INBRE Program of the National Center for Research Resources.

Acknowledgements
We thank additional undergraduate students who contributed to the project, including M. Mclaughlin, Haddy Faal and Bailey Johnson.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.