Microbial Pathogenesis 169 (2022) 105636

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Contribution of *Streptococcus pyogenes* M87 protein to innate immune resistance and virulence

Yujiro Hirose [a,b,*], Piotr Kolesinski [c], Masanobu Hiraoka [b,d], Satoshi Uchiyama [b], Raymond H. Zurich [b], Monika Kumaraswamy [e,f], Elisabet Bjanes [b], Partho Ghosh [c], Shigetada Kawabata [a,**], Victor Nizet [b,g]

[a] Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, 5650871, Japan
[b] Department of Pediatrics, University of California at San Diego, La Jolla, CA, 92093, USA
[c] Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA, 92093, USA
[d] Department of Otorhinolaryngology-Head and Neck Surgery, Wakayama Medical University, Wakayama, Wakayama, 6418509, Japan
[e] Department of Medicine, University of California at San Diego, La Jolla, CA, 92093, USA
[f] Infectious Diseases Section, VA San Diego Healthcare System, San Diego, CA, 92161, USA
[g] Skaggs School of Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA, 92093, USA

ARTICLE INFO

Keywords:
Streptococcus pyogenes
emm87
M protein
Virulence factor

ABSTRACT

Streptococcus pyogenes is a pre-eminent human pathogen, and classified by the hypervariable sequence of the *emm* gene encoding the cell surface M protein. Among a diversity of M/*emm* types, the prevalence of the M/*emm*87 strain has been steadily increasing in invasive *S. pyogenes* infections. Although M protein is the major virulence factor for globally disseminated M/*emm*1 strain, it is unclear if or how the corresponding M protein of M/*emm*87 strain (M87 protein) functions as a virulence factor. Here, we use targeted mutagenesis to show that the M87 protein contributes to bacterial resistance to neutrophil and whole blood killing and promotes the release of mature IL-1β from macrophages. While deletion of *emm87* did not influence epithelial cell adherence and nasal colonization, it significantly reduced *S. pyogenes*-induced mortality and bacterial loads in a murine systemic infection model. Our data suggest that *emm87* is involved in pathogenesis by modulating the interaction between *S. pyogenes* and innate immune cells.

1. Background

Streptococcus pyogenes causes diverse diseases in humans, ranging from common superficial throat and skin infections to invasive conditions, including septicemia, cellulitis, puerperal fever, necrotizing fasciitis, and streptococcal toxic shock syndrome [1,2]. According to estimates, there are 111 million cases of streptococcal impetigo and 616 million cases of streptococcal pharyngitis annually worldwide, with 663,000 cases of invasive infection [3]. The careful characterization of emerging *S. pyogenes* strains provides a critical knowledge base for responding to future epidemics caused by this pathogen.

The M protein, encoded by the *emm* gene, allows classification of *S. pyogenes* into at least 200 *emm* sequence types distributed throughout the world [4–7]. M protein is the most abundant protein anchored to the *S. pyogenes* cell surface, and it serves diverse functions in disease pathogenesis [2,8–10]. Numerous studies have shown that various *S. pyogenes* strains utilize their serotype-specific M proteins for adherence to and internalization into pharyngeal epithelial cells or keratinocytes [11–14]. M protein can also play a key role in *S. pyogenes* survival in human blood [15] by impairment of complement-mediated opsonization and avoidance of neutrophil and macrophage phagocytosis [16]. In severe invasive disease, M protein can also induces pathological inflammation mediated by pathological coagulation [17], neutrophil degranulation [18,19], and/or macrophage pyroptosis [20].

Several studies have established correlations between specific *emm* types and disease manifestations [21–24]. Recently, isolates of M/*emm*87 have been associated with familial and hospital clusters of invasive *S. pyogenes* infections and thus proposed to be highly transmissible [25,26]. The prevalence of M/*emm*87 has been steadily increasing among invasive *S. pyogenes* infections in Portugal [27], Spain

* Corresponding author. Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, 5650871, Japan.
** Corresponding author. Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, 5650871, Japan.
E-mail addresses: yujirohirose@dent.osaka-u.ac.jp (Y. Hirose), kawabata@dent.osaka-u.ac.jp (S. Kawabata).

https://doi.org/10.1016/j.micpath.2022.105636
Received 3 December 2021; Received in revised form 16 May 2022; Accepted 10 June 2022
Available online 17 June 2022
0882-4010/© 2022 Published by Elsevier Ltd.

[28], and France [29], and it now ranks fifth in Europe [30] and thirteenth in the United States [31] in recent invasive disease surveillance. Furthermore, based on genomic analysis, M/*emm*87 strains are predicted to be capable of future global pandemic spread [32].

The unique sequence characteristics of each M protein/*emm* type can determine its spectrum of phenotypic manifestations and overall contribution to disease pathogenesis, but this information is not currently available for *M/emm87* strains. In this study, we used targeted mutagenesis coupled with human tissue culture systems and murine challenge models to characterize potential virulence role(s) the M87/*emm87* protein.

2. Methods

2.1. Bacterial strains and culture conditions

Streptococcus pyogenes M87 20161436 (SRA accession: SAMN07154152) is an invasive disease isolate from Minnesota that possesses the hyaluronic acid capsule and exotoxin genes *speC*, *speG*, *speJ*, *ssa*, and *smeZ* [31]. *S. pyogenes* were grown at 37 °C in screw-cap centrifuge tubes (Falcon) filled with Todd-Hewitt broth supplemented with 0.2% yeast extract (THY broth) (Hardy Diagnostics) in an ambient atmosphere without shaking. For experiments, overnight cultures of *S. pyogenes* were diluted 1:50 into fresh THY broth and colony forming units (CFU) determined by dilution plating on THY agar. *Escherichia coli* MC1061 was used as a host for derivatives of plasmids pHY304 [33] and pDCerm [34], while *E. coli* BL-21 Gold (DE3) was used for derivatives of modified pET28b vector (Novagen). *E. coli* were cultured in Luria-Bertani (LB) medium (Hardy Diagnostics) at 37 °C with agitation. For selection and maintenance of strains, antibiotics were added to the medium at the following concentrations: erythromycin, 500 μg/mL for *E. coli* and 2 μg/mL for *S. pyogenes*; chloramphenicol, 2 μg/mL for *S. pyogenes*; kanamycin (30 μg/ml) for *E. coli*.

2.2. Construction of emm87 mutant, complemented strains, and GFP-expressing strains

An in-frame M87 protein deletion mutant (Δ*emm87*) in the *S. pyogenes* strain 20161436 (WT) background was constructed using the pHY304 temperature-sensitive shuttle knockout vector, using a previously described method [35]. Briefly, a fusion DNA fragment of sequences upstream of the *emm87* gene, the chloramphenicol acetyltransferase gene (*cat*), and sequences downstream of the *emm87* gene were linked by overlapping PCR and used to construct the recombinant pHY304-M87KO plasmid, which was electroporated into the WT M87 parent strain and grown in the presence of erythromycin. Plasmid integration into the chromosome was then selected as a single crossover event at 37 °C, after which culture at 30 °C without antibiotics was undertaken to allow the second crossover and allelic replacement. The deletion of *emm87* was confirmed by site-specific PCR using purified genomic DNA. For construction of a complemented strain, an M87 protein-expressing vector (pM87) was constructed by cloning of the *emm87* gene into shuttle expression plasmid pDCerm [34]. Primers are listed in Table 1. To construct GFP-expressing bacteria, the green fluorescence protein (GFP) expression vector pDCermGFP [36] was transformed into the WT or Δ*emm87* strains.

2.3. Cell culture

Human keratinocyte cell line HaCaT and the human pharyngeal epithelial cell line Detroit 562 were cultured in Dulbecco's Modified Eagle Media (DMEM, Cat#: 10-013-CV, Corning) and Roswell Park Memorial Institute 1640 media (RPMI, Cat# 11875093, Thermo Fisher Scientific), respectively, supplemented in each case with 10% fetal bovine serum (FBS, Cat# 97068-085, VWR International LLC). The human THP-1 monocyte cell line was provided and authenticated by ATCC and cultured in RPMI 1640 medium supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 0.2% D-glucose, 10 mM HEPES and 1 mM sodium pyruvate. Cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C to around 70% confluence. To subculture cells, adherent cells were rinsed with PBS without Ca^{2+} or Mg^{2+}, and detached by using trypsin/EDTA solution for ~10 min. Fresh culture medium was added to the cells, which were then centrifuged and resuspended in fresh culture medium, and dispensed into new culture vessels.

2.4. S. pyogenes association and invasion assays

Bacterial association with, and invasion into, HaCaT or Detroit 562 cells were quantified as previously described, with minor modifications [37,38]. Briefly, HaCaT or Detroit 562 cells were seeded at 2×10^5 cells into 24-well plates 1 day prior to bacterial infection. Immediately prior to the assay, the culture media of HaCaT and Detroit 562 were replaced with DMEM +2% FBS and RPMI +2% FBS, respectively. Log-phase bacteria were used to infect cells at a multiplicity of infection (MOI) of 10 bacteria to 1 cell. Plates were centrifuged for 5 min at 500×*g* to initiate bacterial contact and incubated for 30 min at 37 °C in 5% CO_2 (adherence) or 2 h (invasion). At 30 min after infection, GFP-expressing strains were imaged with an Axio Observer D1 microscope (Zeiss, Göttingen, Germany). For invasion assays, cells were washed three times with PBS, fresh medium containing 10 μg/ml penicillin plus 100 μg/ml

Table 1
Primers used in this study.

For deletional mutagenesis		
Primer	Sequence (5′-3′)	Purpose
M87KOupF	CCCAAGCTTGCATGAGTTGACCGACTTGC	Construction of pHY304-M87KO
M87KOupR	CCAGTGATTTTTTTCTCCATTATTTGCTCCTTATTTTTC	Construction of pHY304-M87KO
M87KOdownF	GAGTGGCAGGGCGGGGCGTAAAATATAAAAGGGATCAATGATG	Construction of pHY304-M87KO
M87KOdownR	CCAATGCATTGGTTCTGCAGCCGTACCGGTTTTTAGTTTCCG	Construction of pHY304-M87KO
M87KOcatF	ATGGAGAAAAAAATCACTGGATATACC	Construction of pHY304-M87KO
M87KOcatR	TTACGCCCCGCCCTGCCACTCATCGCA	Construction of pHY304-M87KO
M87KOconfirmF	GGCTATCAACGGATGACTCC	Confirmation of *emm87* deletion
M87KOconfirmR	AGCATCAATGCCATCTTGTAA	Confirmation of *emm87* deletion
For construction of plasmid used for ectopic expression of M87 protein (pM87)		
Primer	**Sequence (5′-3′)**	**Purpose**
M87F + pDCerm	CGGAATTCCTGGTCTTTACCTTTTACCGC	Construction of pM87
M87R + pDCerm	CGGGATCCATTGATCCCTTTTATATTTTAG	Construction of pM87
pDCermF + M87	CGGGATCCAAGCTTAGCATGCGCTGAAGCG	Construction of pM87
pDCermR + M87	CGGAATTCGGTACCGCATGCAACCTCTG	Construction of pM87
pDCermUinivF	GAAGAAAAGAGCTTTGCTAGG	Confirmation of pM87
pDCermUinivR	ATCTCCAATCATAAAAATAAC	Confirmation of pM87

gentamicin was added, and cells were incubated for an additional 2 h. After incubation, cells were washed 5 times with PBS, trypsinized and lysed with 0.025% Triton X-100, and plated on THY agar for CFU enumeration.

2.5. Recombinant M87 protein purification

Coding sequences from mature M87 protein (residues 42–330) were cloned into a modified pET28b vector that contained sequences encoding an N-terminal His_6-tag followed by a PreScission protease cleavage site. *E. coli* BL-21 Gold (DE3) transformed with the M87-pET28b vector were grown at 37 °C in LB supplemented with kanamycin, induced at mid-log growth phase with 1 mM isopropyl β-D-1-thiogalactopyranoside, and grown for a further 16 h at 20 °C. Bacteria were then pelleted by centrifugation (2500×*g*, 10 min, RT), re-suspended in 300 mM NaCl, 20 mM sodium phosphate buffer, pH 7.5 (PS buffer) supplemented with 1 mM PMSF, and lysed with a C-5 Emulsiflex. The lysate was clarified by centrifugation (20,000×*g*, 30 min, 4 °C) and loaded onto a column containing Ni^{2+}-NTA resin (Thermo fisher Scientific). Bound proteins were eluted with PS buffer containing 150 mM imidazole, pH 8.0. The eluate was exchanged by dialysis into 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.5 at 4 °C. The His_6-tag was removed from M87 by overnight digestion at 4 °C with PreScission protease. Flow through and wash fractions, which contained the cleaved M87 protein, were pooled together and concentrated with an Amicon centrifugal filter unit and a 10,000 kDa cut-off membrane. Concentrated M87 protein was purified further by gel filtration chromatography using a Superdex 200 16/60 column that had been pre-equilibrated with and run in 100 mM NaCl, 20 mM HEPES pH 7.5.

2.6. Infection of macrophages with S. pyogenes, and LDH and IL-1 β signaling assays

THP-1 monocytes were seeded at 5×10^5 cells in a 24-well plate and differentiated into macrophages for 24 h with 25 nM phorbol myristate acetate (PMA) (Thermo Fisher Scientific). Macrophages in assay media (RPMI-2% FBS) were infected with *S. pyogenes* (exponential phase bacteria, MOI 10) or incubated with the indicated concentration of recombinant M87 protein. Plates were centrifuged at 500×*g* for 5 min to facilitate bacterial contact with macrophages. After 1 h incubation at 37 °C, cells were washed 5 times with PBS, trypsinized and lysed with 0.025% Triton X-100, and plated on THY agar for CFU enumeration (adherence). In addition, supernatants were collected, centrifuged, and analyzed for both LDH release, using the Cytotox 96 Non-Radioactive Cytotoxicity Assay kit (Promega), and IL-1β signaling, as follows: Stably transfected HEK-Blue IL-1β reporter cells (InvivoGen) (40,000 cells per well in 96-well plates), were stimulated at 37 °C in 5% CO_2 with 50 μL of supernatant from infected cells. After 18 h stimulation, supernatants from HEK-Blue cells were analyzed for secreted alkaline phosphatase activity (SEAP) by the addition of 50 μL of supernatant into 150 μL of Quanti-Blue reagent (InvivoGen) and monitoring the optical density at 620 nm via an EnSpire plate reader (PerkinElmer). Finally, for the detection of intracellular bacteria, cells were washed, treated with antibiotics, and incubated for 2 h. After incubation, cells were washed, trypsinized, lysed, and plated for enumeration.

2.7. Bactericidal assays with human blood, serum, and neutrophils

Human whole blood and serum isolated was freshly collected from healthy donors under a protocol approved by the University of California San Diego (UCSD) Human Research Protections Program. Neutrophils were isolated from fresh human blood by density gradient centrifugation using Polymorphprep (Alere Technologies). For the blood bactericidal assay, heparinized human blood (190 μL) and exponential phase bacteria (1.5×10^5 CFU in 10 μL of PBS) were mixed. In serum bactericidal assays, human serum (95 μL) and exponential phase bacteria (1.5×10^4 CFU in 5 μL of PBS) were mixed. In neutrophil killing assays, exponential phase bacteria (2×10^6 CFU/100 μL) were combined with human neutrophils (2×10^5 cells/100 μL) in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific). The mixture was incubated in 96-well plates at 37 °C with 5% CO_2 for 1 h. Viable cell counts were determined by plating diluted samples on THY agar.

2.8. Oxidative burst assay

Oxidative burst measurements were performed as previously described [39]. Briefly, 2×10^6/mL human neutrophils were loaded with 20 μM 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma Aldrich) in HBSS without Ca^{2+} and Mg^{2+} (Thermo Fisher Scientific) and incubated at 37 °C for 20 min. Neutrophils were centrifuged, resuspended to HBSS with Ca^{2+} and Mg^{2+}, infected with MOI 1 bacteria/cell, and incubated for 20 min at 37 °C in 5% CO_2. Assays were performed using untreated control, and 25 nM PMA as positive control. Fluorescence intensity was quantified on an Enspire Alpha plate reader (PerkinElmer) at 485 nm excitation/530 nm emission. For the detection of fluorescence intensities of DCFDA-loaded neutrophils, cells were washed with PBS and subjected to FACSCalibur flow cytometry (BD Biosciences).

2.9. Murine nasal colonization and systemic infection studies

All mouse experiments were conducted in accordance with animal protocols approved by University of California San Diego Institutional Animal Care and Use Committee (IACUC). For nasal colonization studies, 11-week-old female C57BL/6J mice (The Jackson Laboratories) were inoculated intranasally with 16 μL volume of PBS containing 1.6×10^8 CFU of the WT M87 *S. pyogenes* strain, Δ*emm87* mutant, or Δ*emm87*+pM87 complemented strain. After 24 h, mice were sacrificed, nasal tissue homogenized and vortexed with beads in 100 μL of PBS, and CFU were enumerated on sheep blood agar after appropriate dilution. Occasional contaminants were excluded during counting of the CFU by morphology, state of hemolysis, or color of the bacterial colony.

Intraperitoneal infections were performed as reported with minor modifications [40]. Eight-week-old male CD1 mice (Charles River Laboratories) were intraperitoneally injected with 3×10^8 CFU in 200 μL of PBS. Mouse survival was monitored for 14 days. To assess bacterial burden, at 24 h after infection, mice were humanely euthanized with CO_2 followed by cervical dislocation, and blood, kidney, spleen, and liver samples obtained for CFU enumeration. Bacterial counts in blood and homogenates of tissues were determined after plating serial dilutions, with those in the tissues corrected for differences in blood volume or tissue mass.

2.10. Sequence comparison among M proteins

The BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (https://www.genome.jp/tools-bin/clustalw) algorithms were used to compare the sequences of the hypervariable region (HVR) or C-repeat region of M proteins. The sequences of the HVR were extracted as the first 50 amino acids of the mature form of the protein, after the signal peptide sequence. Since the C-repeat domain is highly conserved among M proteins [41], C-repeat region in *S. pyogenes* M1 SF370 strain (GenBank: AE004092.2) was used to identify the C-repeats regions of M87, M5 (Manfredo) (GenBank: AM295007.1), and M6 (GenBank: AP012335.1) strains.

2.11. Quantification and statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software Inc). Kruskal-Wallis test with Dunn's post hoc test was used for multiple comparisons. Differences between groups were analyzed using a Mann-Whitney *U* test. Mouse survival was analyzed with a log-rank test. Sample sizes and *p* values are indicated in figure

Microbial Pathogenesis 169 (2022) 105636

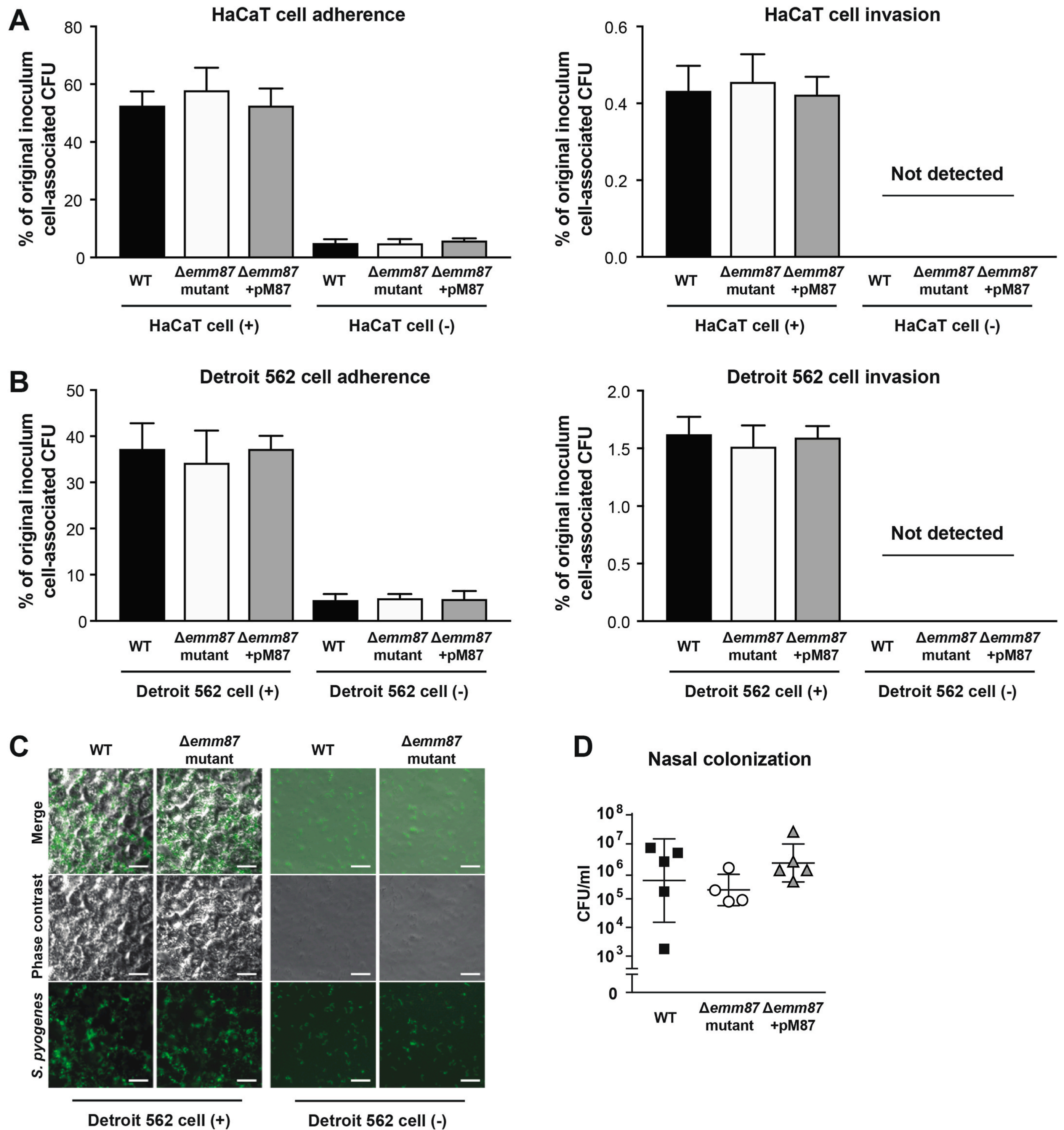

Fig. 1. M87 protein is not a major determinant of epithelial cell adherence and colonization. Efficiency of adherence to and intracellular invasion into HaCaT cells (A) and Detroit 562 cells (B) by *S. pyogenes* WT, Δ*emm87*, and Δ*emm87*+pM87 strains. Total cell association efficiencies were calculated by dividing the CFU value obtained at 30 min after infection by the value of the original inoculum. Invasion efficiencies were calculated by dividing the CFU value obtained at 2 h after antibiotic addition to kill extracellular bacteria by the value of the original inoculum. Vertical lines represent the mean + S.D. Values are presented as the mean of 6 wells from one of 3 independent experiments. (C) Microscopic image of GFP-expressing strains in the association assay using Detroit 562 cells. At 30 min after infection, wells were washed and imaged. (D) Murine nasal cocolonization after 24 h post infection (n = 4, or 5). Vertical lines represent the mean ± geometric S.D. Statistical differences between groups were analyzed using a Mann-Whitney's *U* test.

legends.

3. Results

3.1. M87 protein is not a major determinant of epithelial cell adherence and colonization

To explore potential roles of the M87 protein in *S. pyogenes* pathogenesis, we constructed an isogenic *emm87* deletion mutant strain (Δ*emm87*), and complemented this mutant through transformation with plasmid pM87 (Δ*emm87*+pM87). The M protein is anchored to the *S. pyogenes* cell wall peptidoglycan through a C-terminal LPxTG motif [42,43], and has been reported to function as an epithelial cell adhesin and invasin in certain serotype strains [2]. We performed assays for *S. pyogenes* adherence to and invasion into immortalized lines of human keratinocytes (HaCaT) and human pharyngeal epithelial cells (Detroit 562). Levels of *S. pyogenes* adherence (total cell associated bacteria) and invasion (intracellular CFU protected from gentamicin killing) in HaCaT and Detroit 562 cells were comparable among the WT, Δ*emm87*, and Δ*emm87*+pM87 strains (Fig. 1A and B). Negative control wells that were not seeded with cells showed ~10-fold lower association rates compared to wells that were seeded with cells. In addition, green fluorescent protein (GFP)-expressing strains provided a microscopic image that correlated with results from the Detroit 562 cell association assay (Fig. 1C). Likewise *in vivo*, nasal colonization with GAS was similar at 24 h for WT, mutant, and complemented strains in the mouse intranasal challenge model (Fig. 1D). These data suggest that the M87 protein is not an essential contributor to epithelial cell adherence and colonization by the pathogen.

3.2. *S. pyogenes* M87 protein promotes the release of mature IL-1β from macrophages

Various *S. pyogenes* M proteins including M1 protein elicit macrophage pyroptosis and IL-1β release [20]. We infected THP-1 macrophages with the WT *S. pyogenes,* Δ*emm87* mutant, and complemented strains. At 1 h after infection, levels of *S. pyogenes* adherence (total cell associated bacteria) to THP-1 macrophages were comparable among the WT, Δ*emm87*, and Δ*emm87*+pM87 strains (Fig. 2A). However, release of intracellular LDH in the macrophages at 1 h post-infection was lower for the Δ*emm87* strain in a cytotoxicity assay (Fig. 2B). To investigate whether M87 protein induces cell death in THP-1 macrophages directly, we purified mature recombinant M87 protein (residues 42–330). In an LDH release assay, M87 protein yielded a dose-dependent increase in macrophage cytotoxicity (Fig. 2B). Pyroptotic macrophage cell death is accompanied by inflammasome activation and mature IL-1β release [44]. To allow functional discrimination between mature IL-1β and pro-IL-1β, which is not possible by ELISA, we assessed functional signaling activity using HEK-Blue IL-1β reporter cells. Release of mature (signaling competent) IL-1β from THP-1 macrophages cells was decreased in Δ*emm87* strain compared to the WT and Δ*emm87*+pM87 complemented strains (Fig. 2C), a finding corroborated by dose-dependent mature IL-1β induction by recombinant M87 protein (Fig. 2C). Together, these results indicate that the *S. pyogenes* M87

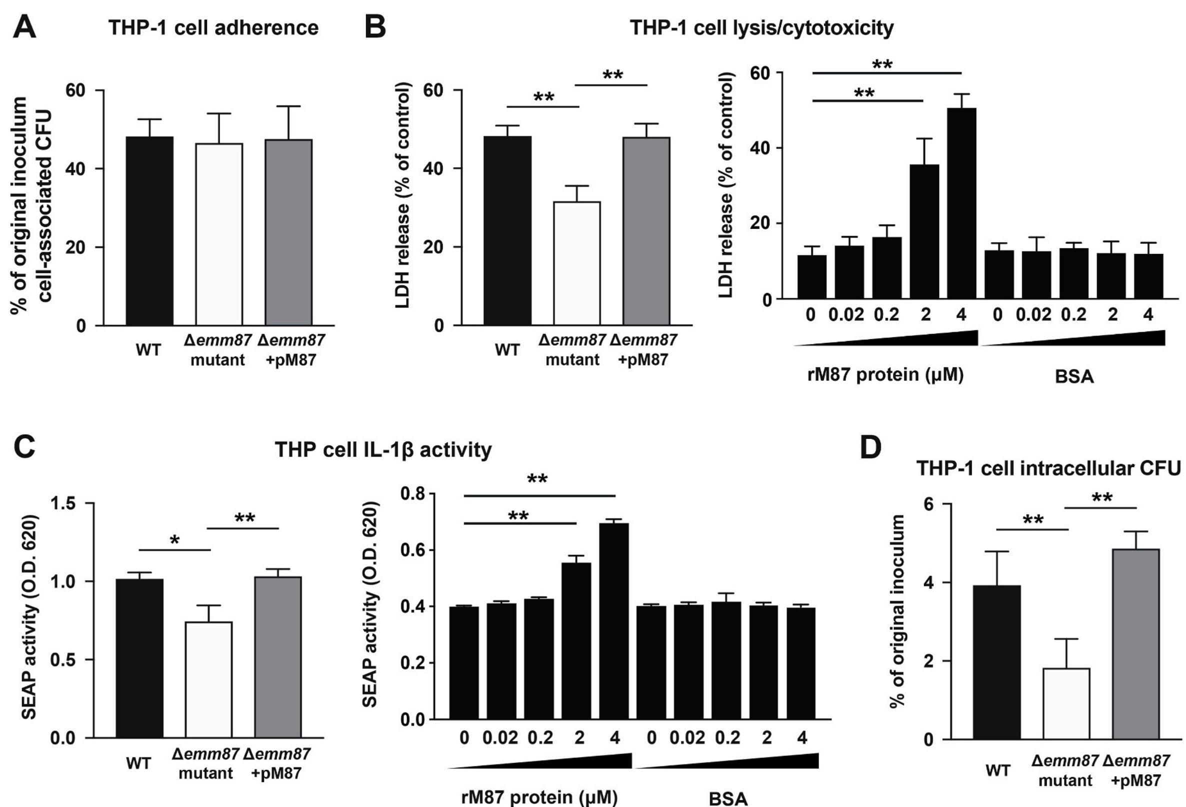

Fig. 2. M87 protein contributes to the release of mature IL-1β from THP-1 macrophage. THP-1 macrophages were infected with *S. pyogenes* (MOI 10) or incubated with the indicated concentration of recombinant M87 protein (rM87 ptotein). At 1 h post-infection or incubation, levels of *S. pyogenes* adherence to THP-1 macrophages were detected, and supernatants were collected for assessing LDH release and IL-1β signaling. For detection of intracellular bacteria, cells were treated with antibiotics for an additional 2 h. (A) Efficiency of adherence to THP-1 macrophages by *S. pyogenes* strains. Total cell association efficiencies were calculated by dividing the CFU value obtained at 1 h after infection by the value of the original inoculum. (B) Detection of LDH in culture supernatants. The control, 100% release of LDH from THP-1 macrophage, were induced by using lysis buffer. (C) Quantification of mature IL-1β in culture supernatants. Secreted alkaline phosphatase activity (SEAP) from HEK-Blue IL-1β reporter cells were quantified. (D) Efficiency of intracellular *S. pyogenes* survival after treatment of the well with extracellular antibiotic. Vertical lines represent the mean + S.D. Values are presented as the mean of 6 wells from one of 3 independent experiments. Statistical differences between groups were analyzed using a Kruskal-Wallis test with Dunn's post hoc test. $**p < 0.01$. $*p < 0.05$. Bovine serum albumin (BSA): Negative control.

protein, in a manner like the M1 protein, can trigger the release of mature IL-1β from macrophages.

In addition, we also observed that the absence of *emm87* led to decreased recovery of intracellular CFU in the gentamicin protection assay (Fig. 2D), suggesting that *emm87* contributes to bacterial survival in THP-1 macrophages.

3.3. M87 protein contributes to S. pyogenes resistance to whole blood and neutrophil killing

Increasing epidemiologic association with invasive infection denotes a capacity for M87 *S. pyogenes* strains to enter the bloodstream and disseminate to deeper tissues. This in turn implies a relative resistance to antimicrobial properties of human blood, such as the opsonophagocytic clearance of bacteria by neutrophils, the most abundant circulating leukocyte. To investigate the contribution of M87 protein to resistance to immune clearance, we measured bacterial survival upon incubation with human neutrophils, blood, and serum. Loss of the *emm87* gene reduced *S. pyogenes* survival in whole blood and neutrophil killing assays, but it did not sensitize the bacterium to killing by serum, to which it is intrinsically resistant (Fig. 3A). The neutrophil bactericidal assay was performed in HBSS without serum as assay medium. In addition, serum humoral factors did not seem to affect the result of the whole blood bactericidal assay. These results suggest that an interaction with humoral immunity and/or complement is not required for M87 protein to provide resistance to neutrophil bactericidal activity. Of note, infection with the *emm87* deletion mutant resulted in higher neutrophil oxidative burst as compared to *S. pyogenes* WT and Δ*emm87*+pM87 strains (Fig. 3B and C), likely contributing to the enhanced bactericidal activity; however, recombinant M87 protein itself was insufficient to suppress the PMA-induced neutrophil oxidative burst (Fig. 3D).

3.4. M87 protein contributes to S. pyogenes virulence in systemic infection

To assess the role of M87 protein in systemic infection, mice were infected intraperitoneally with the *S. pyogenes* strains. In this infection model, the *emm87* deletion mutant produced significantly reduced mortality compared to the WT or Δ*emm87*+pM87 strains (Fig. 4A). These results were corroborated upon examination of bacterial burden in blood, kidney, spleen, and liver samples obtained 24 h after intraperitoneal infection (Fig. 4B). In all these tissues, the numbers of CFUs obtained from the WT strain-infected and Δ*emm87*+pM87 strain-infected mice were significantly higher compared to the CFU counts seen in the *emm87* deletion mutant-infected mice. These results suggest that M87 protein functions as a virulence factor by resisting innate immune bacterial clearance during systemic infection.

3.5. Sequence homology among M proteins

It has been reported that not only the hypervariable region (HVR) of M proteins but also the C-repeat region of M proteins contributes to adherence of *S. pyogenes* of the M6 serotype [45], and M1 and M5

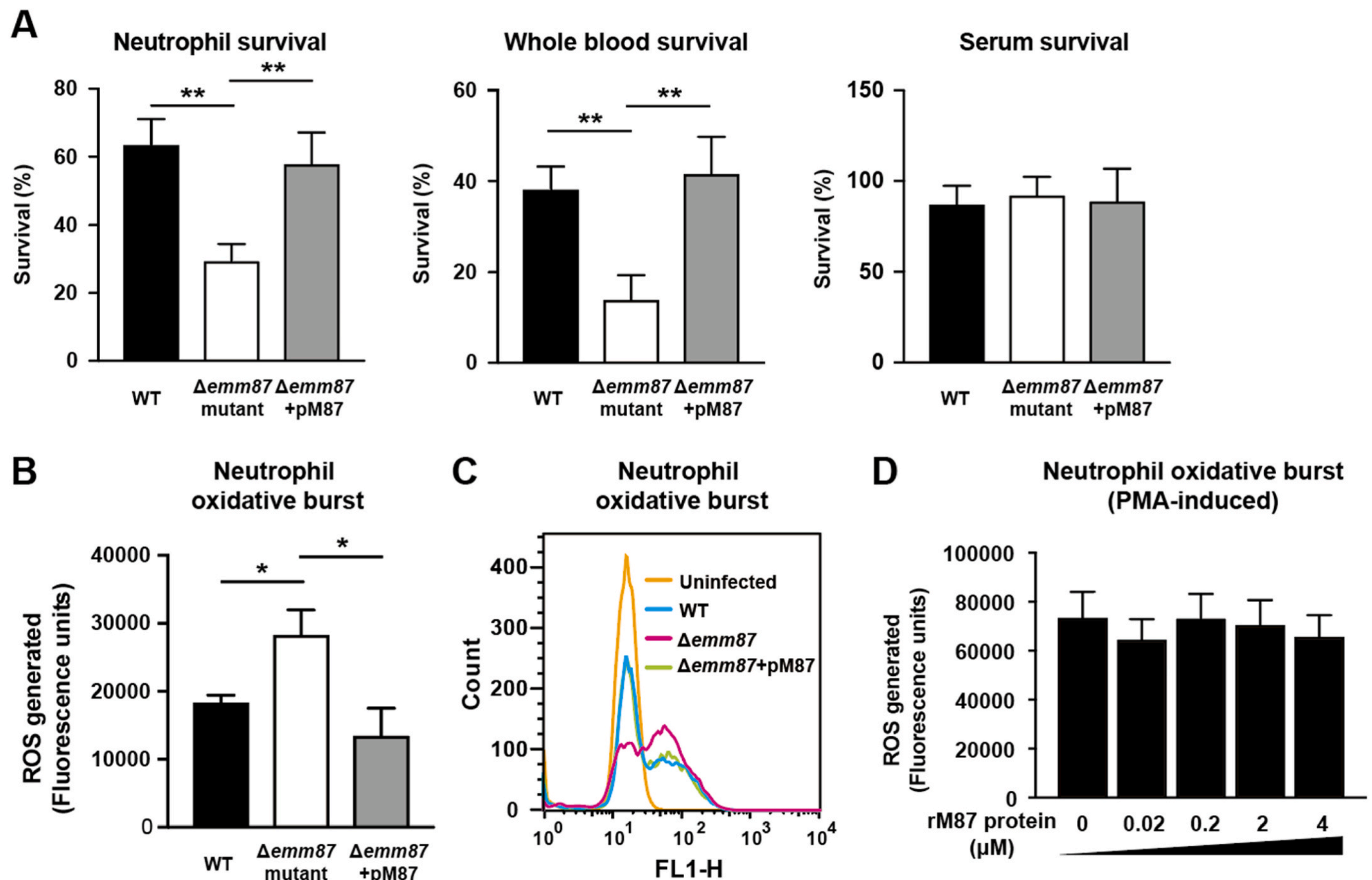

Fig. 3. M87 protein contributes to *S. pyogenes* resistance to whole blood and neutrophil killing. Bacteria were incubated with human neutrophils, in heparinized blood, or in serum at 37 C° for 1 h in a 5% CO_2 atmosphere. (A) Bactericidal activity of neutrophils, blood, and serum. Survival rate was calculated by dividing the CFU value after the period of incubation by the CFU value of the original inoculum. (B) Neutrophil oxidative burst, as measured by the fluorescence intensity of DCFDA-loaded neutrophils. (C) Representative flowcytometric analysis of DCFDA-loaded neutrophils. (D) Relationship between PMA-induced neutrophil oxidative burst and concentration of rM87 protein. Vertical lines represent the mean + S.D. Values are presented as the mean of 6 wells from one of 3 independent experiments. Statistical differences between groups were analyzed using a Kruskal-Wallis test with Dunn's post hoc test. $^{**}p < 0.01$. $^{*}p < 0.05$.

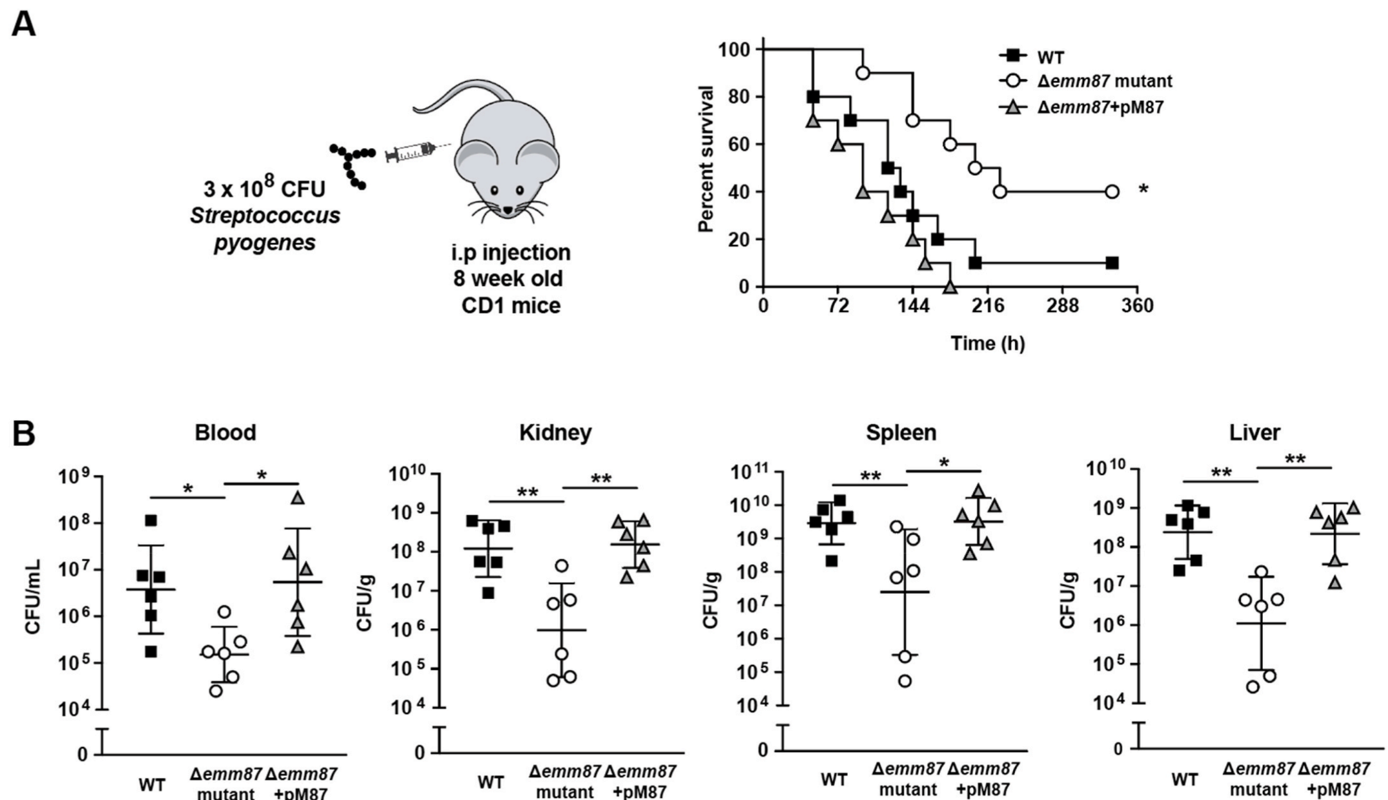

Fig. 4. M87 protein contributes to *S. pyogenes* virulence in systemic infection. (A) Survival rates of mice after intraperitoneal infection. CD-1 mice were infected with the *S. pyogenes* WT, Δ*emm87*, or Δ*emm87*+pM87 strains (~3.0 × 10^8 CFU in 200 μL of PBS) (n = 10). Mouse survival was monitored for 14 days. Statistical differences between groups were analyzed using a log-rank test. *$p < 0.05$ versus WT strain-infected mice. (B) Bacterial burden in the blood, kidney, spleen, and liver after 24 h of infection (n = 6). Vertical lines represent the mean ± geometric S.D. Statistical differences between groups were analyzed using a Mann-Whitney's *U* test. *$p < 0.05$ and **$p < 0.01$.

Table 2
Sequence comparison among M proteins in hypervariable region (HVR).

	Blastp (protein-protein BLAST)						ClustalW
Description	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Aligned. Score
M87_HVR vs M1_HVR	23.1	23.1	78%	2.00E-05	32.56	50	22
M87_HVR vs M5_HVR	No significant similarity found.						18
M87_HVR vs M6_HVR	No significant similarity found.						14

Table 3
Sequence comparison among M proteins in C-repeat region.

	Blastp (protein-protein BLAST)						ClustalW
Description	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Aligned. Score
M87_C_repeat vs M1_C_repeat	103	155	94%	3.00E-36	73.42%	84	69.05
M87_C_repeat vs M5_C_repeat	90.9	167	96%	1.00E-30	67.90%	111	63.10
M87_C_repeat vs M6_C_repeat	107	193	98%	9.00E-38	76.54%	84	73.81
M1_C_repeat vs M6_C_repeat	119	190	100%	2.00E-42	81.25%	84	77.38
M1_C_repeat vs M5_C_repeat	83.6	163	94%	1.00E-27	62.50%	84	57.14
M5_C_repeat vs M6_C_repeat	112	112	69%	5.00E-39	77.38%	84	73.81

serotypes [46]. Therefore, we compared the sequence of the HVR (Table 2) and C-repeat region (Table 3) among M87, M1, M5, and M6 proteins. With BLASTP search, the similarity to the HVR of M87 protein was detected only within the HVR of M1 protein. On the other hand, high homologies of the C-repeat region among M87, M1, M5, and M6 proteins were observed.

4. Discussion

S. pyogenes strains are commonly typed based on the N-terminal hypervariable region of the *emm* gene (https://www.cdc.gov/streplab/groupa-strep/emm-background.html). M proteins can assume a variety of virulence functions depending on the specific M type [2], but whether and how the M87 protein might function as a virulence factor had not been studied. Here, we show that the *S. pyogenes* M87 protein contributes to resistance to neutrophil and whole blood killing, and to the

release of mature IL-1β from macrophages. *In vivo*, loss of the *emm87* gene decreases bacterial virulence in systemic infection, supporting a model in which *S. pyogenes* M87 protein promotes pathogenesis by influencing the interaction between *S. pyogenes* and innate immune cells.

In contrast, we found that *S. pyogenes* M87 protein does not contribute to microbial colonization, neither *in vitro* nor *in vivo*. This result contrasts with reports in which various M proteins protein can function as an epithelial adhesin and invasin [2]. This further contrasts with the reduction in pharyngeal cell adherence and colonization phenotypes observed in a wild-type *S. pyogenes* M1T1 strain compared to its isogenic M1 protein deletion mutant [47]. These variations suggest that strain-specific differences in the *emm* gene, along with the full complement of other potential surface adhesins expressed by an individual *S. pyogenes* strain, influence the particular role of a given M protein to bacterial binding to host skin and mucosal epithelium.

M87 protein, like M1 protein, induced the release of mature IL-1β from macrophages in a dose-dependent manner [20], and the *emm87* deletion mutant was significantly more susceptible in a neutrophil killing assay as compared to WT and Δ*emm87*+pM87 strains. The host's initial barrier against *S. pyogenes* infection is the physical integrity of mucosal or skin epithelium and the beneficial antagonism of commensal members of the microbiome. However, once *S. pyogenes* has gained a deeper foothold in the host, the innate immune system, including macrophages and neutrophils, becomes central to defense against invasive infection. In our results, at 24 h after intraperitoneal infection in mice, WT- and Δ*emm87*+pM87-strain infected mice showed higher bacterial burden in blood, kidney, spleen, and liver samples compared to *emm87* deletion mutant-infected mice, showing that the M87 protein impedes systemic clearance of bacteria by innate immunity.

M87 contributed to *S. pyogenes* resistance to whole blood and neutrophil killing independent of the efficiency of phagocytic uptake. In blood, opsonophagocytosis involves interaction between *S. pyogenes* and proteins present in plasma such as complement and IgG [15]. On the other hand, *S. pyogenes* is not killed by serum alone since the complement membrane attack complex is not effective in killing Gram-positive bacterial species due to their thick cell walls [48]. In this study, *S. pyogenes* M87 protein did not affect bacterial viability in serum alone, but rather contributed to neutrophil bactericidal activity without plasma-mediated opsonization. We also found that the *emm87* deletion mutant was associated with significantly increased neutrophil ROS production compared to WT and Δ*emm87*+pM87 strains. This contrasts with previous findings showing the lack of influence by *emm1* on neutrophils oxidative burst [39]. Considering the result that recombinant M87 protein did not suppress the PMA-induced neutrophil oxidative burst, the *emm87* deletion may affect cell surface morphology or distribution of surface molecules, possibly decreasing the interaction between the neutrophils and streptolysin O [39].

M/*emm87* have been proposed to be highly transmissible and associated with familial and hospital clusters of invasive *S. pyogenes* infections [25,26]. In this study, despite the high homology of the C-repeat regions that contributes to the adherence to epithelial cells [45,46], M87 protein is not a major determinant of epithelial cell adherence and colonization. This discrepancy suggests that M/*emm87* strains possess other surface determinants that contribute more strongly to host colonization. The pathogenicity exerted by the M87 protein may be derived from the HVR. The HVR sequence of M87 protein more similar to that of M1 protein than to those of M5 and M6 proteins. The M1/*emm1* strain is the most prevalent worldwide [7], and the HVR of M1 protein is important for its virulence [49,50]. Further verification is needed to clarify the region(s) of M87 protein that contributes to its pathogenicity.

In summary, our findings suggest that *S. pyogenes emm87* is a virulence factor necessary for resistance to innate immune cells, including macrophages and neutrophils. M/*emm*87 strains are increasingly associated with invasive infections and expected to expand their prominence soon based on epidemiologic and genetic studies. Our findings provide a first step in devising preventive strategies against *S. pyogenes emm87* strains.

Funding

This study was supported in part by AMED (JP21fk0108044, JP21fm0208007), Japanese Society for the Promotion of Science (JSPS) KAKENHI (grant numbers 20K18474), JSPS Overseas Research Fellowships, and NIH R01 Grants AI154149 and AI144901. The funders had no role in study design, data collection or analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Yujiro Hirose: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Piotr Kolesinski:** Writing – review & editing, Investigation, Formal analysis. **Masanobu Hiraoka:** Writing – review & editing, Investigation, Formal analysis. **Satoshi Uchiyama:** Writing – review & editing, Investigation, Formal analysis. **Raymond H. Zurich:** Writing – review & editing, Investigation, Formal analysis. **Monika Kumaraswamy:** Writing – review & editing, Investigation, Formal analysis. **Elisabet Bjanes:** Writing – review & editing, Investigation, Formal analysis. **Partho Ghosh:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Shigetada Kawabata:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis. **Victor Nizet:** Writing – original draft, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yujiro Hirose reports financial support that was provided by the Japanese Society for the Promotion of Science (JSPS). Shigetada Kawabata reports financial support that was provided by Japan Agency for Medical Research and Development (AMED). Partho Ghosh reports financial support that was provided by National Institutes of Health. Victor Nizet reports a relationship with Vaxcyte, Inc. that includes funding of grants.

References

[1] M.W. Cunningham, Pathogenesis of group A streptococcal infections, Clin. Microbiol. Rev. 13 (2000) 470–511.

[2] M.J. Walker, T.C. Barnett, J.D. McArthur, J.N. Cole, C.M. Gillen, A. Henningham, et al., Disease manifestations and pathogenic mechanisms of Group A *Streptococcus*, Clin. Microbiol. Rev. 27 (2014) 264–301.

[3] In, J.J. Ferretti, D.L. Stevens, V.A. Fischetti (Eds.), *Streptococcus Pyogenes* : Basic Biology to Clinical Manifestations. Oklahoma City (OK, 2016.

[4] B. Beall, R. Facklam, T. Thompson, Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci, J. Clin. Microbiol. 34 (1996) 953–958.

[5] R. Facklam, B. Beall, A. Efstratiou, V. Fischetti, D. Johnson, E. Kaplan, et al., Emm typing and validation of provisional M types for group A streptococci, Emerg. Infect. Dis. 5 (1999) 247–253.

[6] M. Sanderson-Smith, D.M. De Oliveira, J. Guglielmini, D.J. McMillan, T. Vu, J. K. Holien, et al., A systematic and functional classification of *Streptococcus pyogenes* that serves as a new tool for molecular typing and vaccine development, J. Infect. Dis. 210 (2014) 1325–1338.

[7] A.C. Steer, I. Law, L. Matatolu, B.W. Beall, J.R. Carapetis, Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development, Lancet Infect. Dis. 9 (2009) 611–616.

[8] S. Oehmcke, O. Shannon, M. Morgelin, H. Herwald, Streptococcal M proteins and their role as virulence determinants, Clin. Chim. Acta 411 (2010) 1172–1180.

[9] D. Metzgar, A. Zampolli, The M protein of group A *Streptococcus* is a key virulence factor and a clinically relevant strain identification marker, Virulence 2 (2011) 402–412.

[10] H.R. Frost, M. Sanderson-Smith, M. Walker, A. Botteaux, P.R. Smeesters, Group A streptococcal M-like proteins: from pathogenesis to vaccine potential, FEMS Microbiol. Rev. 42 (2018) 193–204.

[11] H.S. Courtney, C. von Hunolstein, J.B. Dale, M.S. Bronze, E.H. Beachey, D.L. Hasty, Lipoteichoic acid and M protein: dual adhesins of group A streptococci, Microb. Pathog. 12 (1992) 199–208.

[12] D. Cue, P.E. Dombek, H. Lam, P.P. Cleary, *Streptococcus pyogenes* serotype M1 encodes multiple pathways for entry into human epithelial cells, Infect. Immun. 66 (1998) 4593–4601.
[13] R.P. Ellen, R.J. Gibbons, M protein-associated adherence of *Streptococcus pyogenes* to epithelial surfaces: prerequisite for virulence, Infect. Immun. 5 (1972) 826–830.
[14] N. Siemens, N. Patenge, J. Otto, T. Fiedler, B. Kreikemeyer, *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing, J. Biol. Chem. 286 (2011) 21612–21622.
[15] L. Staali, M. Morgelin, L. Bjorck, H. Tapper, *Streptococcus pyogenes* expressing M and M-like surface proteins are phagocytosed but survive inside human neutrophils, Cell Microbiol. 5 (2003) 253–265.
[16] M. Weineisen, U. Sjobring, M. Fallman, T. Andersson, Streptococcal M5 protein prevents neutrophil phagocytosis by interfering with CD11b/CD18 receptor-mediated association and signaling, J. Immunol. 172 (2004) 3798–3807.
[17] L.I. Pahlman, E. Malmstrom, M. Morgelin, H. Herwald, M protein from *Streptococcus pyogenes* induces tissue factor expression and pro-coagulant activity in human monocytes, Microbiology (Read.) 153 (2007) 2458–2464.
[18] O. Soehnlein, S. Oehmcke, X. Ma, A.G. Rothfuchs, R. Frithiof, N. van Rooijen, et al., Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein, Eur. Respir. J. 32 (2008) 405–412.
[19] H. Herwald, H. Cramer, M. Morgelin, W. Russell, U. Sollenberg, A. Norrby-Teglund, et al., M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage, Cell 116 (2004) 367–379.
[20] J.A. Valderrama, A.M. Riestra, N.J. Gao, C.N. LaRock, N. Gupta, S.R. Ali, et al., Group A streptococcal M protein activates the NLRP3 inflammasome, Nat Microbiol 2 (2017) 1425–1434.
[21] D.E. Bessen, C.M. Sotir, T.L. Readdy, S.K. Hollingshead, Genetic correlates of throat and skin isolates of group A streptococci, J. Infect. Dis. 173 (1996) 896–900.
[22] F. Koutouzi, A. Tsakris, P. Chatzichristou, E. Koutouzis, G.L. Daikos, E. Kirikou, et al., *Streptococcus pyogenes emm* types and clusters during a 7-year period (2007 to 2013) in pharyngeal and nonpharyngeal pediatric isolates, J. Clin. Microbiol. 53 (2015) 2015–2021.
[23] K. Ekelund, J. Darenberg, A. Norrby-Teglund, S. Hoffmann, D. Bang, P. Skinhoj, et al., Variations in *emm* type among group A streptococcal isolates causing invasive or noninvasive infections in a nationwide study, J. Clin. Microbiol. 43 (2005) 3101–3109.
[24] A. Friaes, F.R. Pinto, C. Silva-Costa, M. Ramirez, J. Melo-Cristino, Portuguese Group for the Study of Streptococcal I. Group A streptococci clones associated with invasive infections and pharyngitis in Portugal present differences in *emm* types, superantigen gene content and antimicrobial resistance, BMC Microbiol. 12 (2012) 280.
[25] A.R. Flores, R.A. Luna, J.K. Runge, S.A. Shelburne 3rd, C.J. Baker, Cluster of fatal group A streptococcal *emm87* infections in a single family: molecular basis for invasion and transmission, J. Infect. Dis. 215 (2017) 1648–1652.
[26] M. Montes, E. Tamayo, E. Onate, E.G. Perez-Yarza, E. Perez-Trallero, Outbreak of *Streptococcus pyogenes* infection in healthcare workers in a paediatric intensive care unit: transmission from a single patient, Epidemiol. Infect. 141 (2013) 341–343.
[27] A. Friaes, J. Melo-Cristino, M. Ramirez, Portuguese Group for the Study of Streptococcal I. Changes in *emm* types and superantigen gene content of *Streptococcus pyogenes* causing invasive infections in Portugal, Sci. Rep. 9 (2019), 18051.
[28] P. Villalon, J.A. Saez-Nieto, V. Rubio-Lopez, M.J. Medina-Pascual, N. Garrido, G. Carrasco, et al., Invasive *Streptococcus pyogenes* disease in Spain: a microbiological and epidemiological study covering the period 2007-2019, Eur. J. Clin. Microbiol. Infect. Dis. 40 (2021) 2295–2303.
[29] S. Boukthir, S. Moullec, M.E. Cariou, A. Meygret, J. Morcet, A. Faili, et al., A prospective survey of *Streptococcus pyogenes* infections in French Brittany from 2009 to 2017: comprehensive dynamic of new emergent *emm* genotypes, PLoS One 15 (2020), e0244063.
[30] B. Luca-Harari, J. Darenberg, S. Neal, T. Siljander, L. Strakova, A. Tanna, et al., Clinical and microbiological characteristics of severe *Streptococcus pyogenes* disease in Europe, J. Clin. Microbiol. 47 (2009) 1155–1165.
[31] S. Chochua, B.J. Metcalf, Z. Li, J. Rivers, S. Mathis, D. Jackson, et al., Population and Whole Genome Sequence Based Characterization of Invasive Group A Streptococci Recovered in the United States during 2015. mBio, vol. 8, 2017.
[32] C.E. Turner, M.T.G. Holden, B. Blane, C. Horner, S.J. Peacock, S. Sriskandan, The emergence of successful *Streptococcus pyogenes* lineages through convergent pathways of capsule loss and recombination directing high toxin expression, mBio 10 (2019).
[33] C.A. Pritzlaff, J.C. Chang, S.P. Kuo, G.S. Tamura, C.E. Rubens, V. Nizet, Genetic basis for the β-haemolytic/cytolytic activity of group B *Streptococcus*, Mol. Microbiol. 39 (2001) 236–247.
[34] A. Jeng, V. Sakota, Z. Li, V. Datta, B. Beall, V. Nizet, Molecular genetic analysis of a group A Streptococcus operon encoding serum opacity factor and a novel fibronectin-binding protein, SfbX. J Bacteriol. 185 (2003) 1208–1217.
[35] X. Lauth, M. von Kockritz-Blickwede, C.W. McNamara, S. Myskowski, A. S. Zinkernagel, B. Beall, et al., M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition, J Innate Immun 1 (2009) 202–214.
[36] D. Ly, J.M. Taylor, J.A. Tsatsaronis, M.M. Monteleone, A.S. Skora, C.A. Donald, et al., Plasmin(ogen) acquisition by group A Streptococcus protects against C3b-mediated neutrophil killing, J Innate Immun 6 (2014) 240–250.
[37] Y. Hirose, M. Yamaguchi, K. Goto, T. Sumitomo, M. Nakata, S. Kawabata, Competence-induced protein Ccs4 facilitates pneumococcal invasion into brain tissue and virulence in meningitis, Virulence 9 (2018) 1576–1587.
[38] S. Dohrmann, S. Anik, J. Olson, E.L. Anderson, N. Etesami, H. No, et al., Role for streptococcal collagen-like protein 1 in M1T1 group A *Streptococcus* resistance to neutrophil extracellular traps, Infect. Immun. 82 (2014) 4011–4020.
[39] S. Uchiyama, S. Dohrmann, A.M. Timmer, N. Dixit, M. Ghochani, T. Bhandari, et al., Streptolysin O rapidly impairs neutrophil oxidative burst and antibacterial responses to group A *Streptococcus*, Front. Immunol. 6 (2015) 581.
[40] K.M. Valdes, G.S. Sundar, L.A. Vega, A.T. Belew, E. Islam, R. Binet, et al., The *fruRBA* operon is necessary for group A streptococcal growth in fructose and for resistance to neutrophil killing during growth in whole human blood, Infect. Immun. 84 (2016) 1016–1031.
[41] J. Perez-Casal, N. Okada, M.G. Caparon, J.R. Scott, Role of the conserved C-repeat region of the M protein of *Streptococcus pyogenes*, Mol. Microbiol. 15 (1995) 907–916.
[42] T.C. Barnett, J.R. Scott, Differential recognition of surface proteins in *Streptococcus pyogenes* by two sortase gene homologs, J. Bacteriol. 184 (2002) 2181–2191.
[43] V.A. Fischetti, V. Pancholi, O. Schneewind, Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci, Mol. Microbiol. 4 (1990) 1603–1605.
[44] P. Broz, Immunology: caspase target drives pyroptosis, Nature 526 (2015) 642–643.
[45] N. Okada, M.K. Liszewski, J.P. Atkinson, M. Caparon, 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 (1995) 2489–2493.
[46] I.M. Frick, A. Schmidtchen, U. Sjobring, Interactions between M proteins of *Streptococcus pyogenes* and glycosaminoglycans promote bacterial adhesion to host cells, Eur. J. Biochem. 270 (2003) 2303–2311.
[47] E.L. Anderson, J.N. Cole, J. Olson, B. Ryba, P. Ghosh, V. Nizet, The fibrinogen-binding M1 protein reduces pharyngeal cell adherence and colonization phenotypes of M1T1 group A *Streptococcus*, J. Biol. Chem. 289 (2014) 3539–3546.
[48] E.J. Brown, Interaction of gram-positive microorganisms with complement, Curr. Top. Microbiol. Immunol. 121 (1985) 159–187.
[49] C. McNamara, A.S. Zinkernagel, P. Macheboeuf, M.W. Cunningham, V. Nizet, P. Ghosh, Coiled-coil irregularities and instabilities in group A *Streptococcus* M1 are required for virulence, Science 319 (2008) 1405–1408.
[50] J. Lannergard, M.C. Gustafsson, J. Waldemarsson, A. Norrby-Teglund, M. Stalhammar-Carlemalm, G. Lindahl, The Hypervariable region of *Streptococcus pyogenes* M protein escapes antibody attack by antigenic variation and weak immunogenicity, Cell Host Microbe 10 (2011) 147–157.