The Journal of Immunology

Human IgG Increases Virulence of *Streptococcus pyogenes* through Complement Evasion

David Ermert, Antonin Weckel, Michal Magda, Matthias Mörgelin, Jutamas Shaughnessy, Peter A. Rice, Lars Björck, Sanjay Ram and Anna M. Blom

J Immunol 2018; 200:3495-3505; Prepublished online 6 April 2018;
doi: 10.4049/jimmunol.1800090
http://www.jimmunol.org/content/200/10/3495

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Human IgG Increases Virulence of *Streptococcus pyogenes* through Complement Evasion

David Ermert,*,† Antonin Weckel,* Michal Magda,* Matthias Mörgelin,‡ Jutamas Shaughnessy,† Peter A. Rice,† Lars Björck,‡ Sanjay Ram,† and Anna M. Blom*

***Streptococcus pyogenes* is an exclusively human pathogen that can provoke mild skin and throat infections but can also cause fatal septicemia. This gram-positive bacterium has developed several strategies to evade the human immune system, enabling *S. pyogenes* to survive in the host. These strategies include recruiting several human plasma proteins, such as the complement inhibitor, C4b-binding protein (C4BP), and human (hu)-IgG through its Fc region to the bacterial surface to evade immune recognition. We identified a novel virulence mechanism whereby IgG-enhanced binding of C4BP to five of 12 tested *S. pyogenes* strains expressed diverse M proteins that are important surface-expressed virulence factors. Importantly, all strains that bound C4BP in the absence of IgG bound more C4BP when IgG was present. Further studies with an M1 strain that additionally expressed protein H, also a member of the M protein family, revealed that binding of hu-IgG Fc to protein H increased the affinity of protein H for C4BP. Increased C4BP binding accentuated complement downregulation, resulting in diminished bacterial killing. Accordingly, mortality from *S. pyogenes* infection in hu-C4BP transgenic mice was increased when hu-IgG or its Fc portion alone was administered concomitantly. Electron microscopy analysis of human tissue samples with necrotizing fasciitis confirmed increased C4BP binding to *S. pyogenes* when IgG was present. Our findings provide evidence of a paradoxical function of hu-IgG bound through Fc to diverse *S. pyogenes* isolates that increases their virulence and may counteract the beneficial effects of IgG opsonization. *The Journal of Immunology*, 2018, 200: 3495–3505.**

Streptococcus pyogenes is a commonly encountered and clinically important pathogen (1). Every year, *S. pyogenes* infects ∼700 million people globally and causes life-threatening invasive infections in addition to mild superficial infections, such as impetigo and pharyngitis (1–4). *S. pyogenes* is one of the 10 most fatal human pathogens with ∼500,000 deaths annually (1). In most individuals, *S. pyogenes* affects the skin or oropharynx, but in some instances (∼650,000 cases worldwide annually), *S. pyogenes* invades deeper tissues, causing septicemia and/or necrotizing fasciitis. *S. pyogenes* binds specifically to human plasma proteins and thus evades human immune defenses in particular. Host proteins that bind to *S. pyogenes* include albumin, fibronectin, all four subclasses of IgG and the complement inhibitors C4b-binding protein (C4BP), and factor H (FH) (5–12). Other immune evasion mechanisms include sequestration of cathelicidin, enhanced survival in neutrophil extracellular traps, secretion of proteases and nucleases, and evasion of autophagy that promotes intracellular growth of *S. pyogenes* (13–17).

Complement plays an important role in combating *S. pyogenes* infections. Upon activation, the complement cascade generates inflammatory anaphylatoxins and deposits protein fragments onto foreign surfaces, which enable recognition of pathogens by professional phagocytes (18). Complement activation must be tightly regulated to prevent unwanted damage to host cells, which is achieved by surface-bound as well as soluble complement inhibitors such as C4BP and FH. However, several pathogens, including *S. pyogenes*, have evolved to bind complement inhibitors and evade complement activation to prevent their subsequent elimination (19, 20).

S. pyogenes surface-associated virulence factors include M proteins and M-like proteins such as protein H (21, 22). Although more than 220 variants of the M protein have been identified so far (23), bacteria of the M1 serotype are the most prevalent worldwide (24). Protein H, an IgG Fc−binding virulence factor, presents exclusively on M1-expressing *S. pyogenes* strains and forms complexes with IgG such that IgG cannot activate complement or facilitate opsonophagocytosis, thus rendering them immunologically effete (25, 26). In addition to its ability to bind to several

*Division of Medical Protein Chemistry, Department of Translational Medicine, Lund University, 214 28 Malmo, Sweden; †Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605; and ‡Division of Infection Medicine, Department of Clinical Sciences, Lund University, 221 84 Lund, Sweden

ORCIDs: 0000-0003-4600-9070 (D.E.); 0000-0002-5085-0551 (A.W.); 0000-0003-4857-0769 (M. Magda); 0000-0003-0832-9954 (J.S.); 0000-0002-1348-1734 (A.M.B.).

Received for publication January 23, 2018. Accepted for publication March 12, 2018.

This work was supported by Swedish Research Council Projects 2016-01142 and K2014-58X-07480-29-5, the Swedish Government Funds for Clinical Research, the Torsten Söderberg Foundation, the Crafoord Foundation, the Knut and Alice Wallenberg Foundation, the Lars Hierta Memorial Foundation, the Österlund Foundation, the Gustav V 80-Years Anniversary Foundation, the Gyllenstierna Krapperups Foundation, and National Institutes of Health Grants R01AI114790 (to P.A.R. and S.R.) and R21 AI111728 (to J.S. and S.R.). M. Magda was supported by the Department of Biotechnology at the University of Rzeszow, Poland. A.W. received funding from École Normale Supérieure, Paris, France.

D.E., S.R., and A.M.B. contributed to conceptualization and methodology; D.E., A.W., M. Magda, M. Mörgelin, and J.S. contributed to investigation; D.E., P.A.R., S.R., and A.M.B. contributed to writing the original draft; D.E., A.W., M. Mörgelin, J.S., P.A.R., L.B., S.R., and A.M.B. contributed to writing, reviewing, and editing; D.E., S.R., P.A.R., and A.M.B. contributed to funding acquisition; D.E., M. Mörgelin, J.S., P.A.R., L.B., S.R., and A.M.B. contributed to resources; and D.E., S.R., and A.M.B. contributed to supervision.

The funders had no role in study design, data collection, and interpretation or the decision to submit the work for publication.

Address correspondence and reprint requests to Dr. David Ermert, Division of Medical Protein Chemistry, Department of Translational Medicine, Lund University, The Wallenberg Laboratory, Floor 4, Inga Marie Nilsson's Street 53, Skane University Hospital, 214 28 Malmo, Sweden. E-mail address: David.Ermert@med.lu.se

The online version of this article contains supplemental material.

Abbreviations used in this article: α1AT, α1-antitrypsin; C4BP, C4b-binding protein; FH, factor H; hu, human; PMN, polymorphonuclear cell; tg, transgenic.

serum proteins (6, 7, 21, 26), protein H can also form homodimers (27, 28). Competition between C4BP and human (hu)-IgG for binding to protein H has been suggested (6, 8). In this study, we characterized the interactions between hu- IgG, -C4BP, and -protein H and reported a novel virulence mechanism of *S. pyogenes*.

Materials and Methods

Bacteria and culture conditions

S. pyogenes AP1, AP4, AP8, AP15, AP18 (29), AP28, AP29, AP36, AP38, AP43, AP46, AP60, and AP74 (all from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic) and AP1 isogenic mutants MC25 (M protein$^-$) (30), BM27.6 (protein H$^-$) (25), BM27.6 + pH (31), and BMJ71 (protein H$^-$/M$^-$) (32) were grown in Todd-Hewitt broth overnight at 37˚C and 5% CO_2. Cultures were then diluted to OD_{600} = 0.1 in fresh Todd-Hewitt broth and further incubated at 37˚C in 5% CO_2 and grown to OD_{600} of 0.3–0.4. Prior to use, bacteria were washed with PBS. Strains used are listed in Supplemental Table I.

Proteins and Abs

For flow cytometric analysis, the following Abs were used: mouse anti–hu-C4BP MK104 (33) coupled to biotin; mouse anti–hu-FH MRC OX24 (34) coupled to DyLight 647; goat anti–hu-f(ab)$_2$ (Hycult); donkey f(ab)$_2$ anti-rabbit IgG coupled to AF647 (Jackson ImmunoResearch); donkey f(ab)$_2$ anti-goat IgG coupled to AF647 (Jackson ImmunoResearch); rabbit anti–mouse-C4BP (made in-house) coupled to DyLight 647; and mouse anti–mouse-FH (Hycult) biotin conjugated. Biotin-coupled Abs were stained with streptavidin-PE (eBioscience).

Fab and Fc fragments of hu-IgG were purchased from Calbiochem, and hu-IgG (IVIG; Kiovig) was purchased from Baxalta. Mice were administered pooled hu-Fc fragments (Athens Research), IVIG, or denosumab (Amgen) diluted in PBS. Rabbit IgG was purified from preimmune serum using protein A/G columns. Mouse IgG2a and IgG2b were purchased from ImmunoTools, and goat control IgG was purchased from R&D Biosystems. Goat, rhesus, and cynomolgus IgG were purchased from Nordic Diagnostica. hu-C4BP and FH were purified from human plasma, M18 was purified from culture supernatants using fibrinogen Sepharose, and Enn18 and protein H were expressed and purified from *E. coli*, all according to previously described protocols (8, 35). α1-Antitrypsin (α1AT) was used as a negative control for binding experiments. Plasma-purified C4BP preparations of 2 mg/ml contained between 2 and 10 μg/ml hu-IgG, as determined by a sandwich ELISA for hu-IgG.

Binding of [^{125}I]protein H, [^{125}I]C4BP, [^{125}I]Enn18, and [^{125}I] M18 to purified proteins

Purified proteins (C4BP, FH, α1AT, fibrinogen [American Diagnostica], human serum albumin [Sigma], or fibronectin [Haematologic Technologies]) were diluted to specified concentrations in PBS and immobilized onto microtiter plates (MaxiSorp BreakApart; Nunc) at 4˚C overnight. The plates were washed three times with wash buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% Tween 20), and nonspecific binding sites were blocked with 3% fish gelatin (Norland Products) in wash buffer. ^{125}I-labeled protein H, Enn18, or M18, respectively, were diluted in binding PBS (PBS supplemented with 0.1% Tween 20 and 0.1% BSA) and added in the presence of increasing amounts of IVIG. After incubation at 4˚C overnight and subsequent washing, radioactivity in the wells was detected using a Wizard^2 γ counter (PerkinElmer).

Binding of [^{125}I]C4BP to S. pyogenes

^{125}I-labeled C4BP, diluted in binding PBS, was added to bacteria either in the presence or absence of indicated amounts of IVIG. After 1 h incubation at 37˚C (if not stated otherwise) in 5% CO_2, bacteria were washed three times in 1× PBS, and radioactivity associated with bacteria was detected using a Wizard^2 γ counter (PerkinElmer).

Electron microscopy

The presence and location of individual molecules or as molecular complexes on bacterial surfaces were analyzed by negative staining and transmission electron microscopy, as described previously (36). To visualize protein complexes, C4BP and protein H were coincubated in the presence or absence of 1 mg/ml IVIG for 1 h at 37˚C. To detect C4BP binding to bacteria, C4BP and IVIG were conjugated with colloidal gold (Au). Bacteria were either stained with Ab-Au conjugates or mixed with protein-Au conjugates and incubated for 1 h at 37˚C. Five milliliter aliquots were adsorbed onto carbon-coated grids for 1 min, washed with two drops of water, and stained with two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were examined using a Philips/FEI CM 100 electron microscope operated at an accelerating voltage of 80 kV; images were recorded with an Olympus Soft Imagining Solutions Veleta and side-mounted digital slow scan 2k × 2k CCD camera system using DigitalMicrograph software. The area of protein complexes was measured in Adobe Photoshop CS6. Proteins that were in closer contact than 30 nm or less were considered to interact or to be colocalized. Contrast, brightness, and pseudocolor enhancement were adjusted using Adobe Photoshop CS6.

Survival analysis

All animals were housed and bred under specific pathogen-free conditions in the animal facility at the University of Massachusetts Medical School, Worcester, MA. All experimental groups were sex- and age-matched (6–8-wk-old male and female BALB/c animals and hu-C4BP transgenic [tg] BALB/c).

One day prior to infection, animals were treated either with 1 mg hu-IgG–Fc, 2 mg IVIG or a monoclonal hu-IgG (denosumab), a hu-IgG2 monoclonal Ab that only recognizes human but not mouse RANKL) (37). As negative controls, either sterile PBS or 2 mg goat IgG was used. Animals were infected intravenously via lateral tail vein injection with 100 μl bacterial suspensions in PBS containing *S. pyogenes* AP1 at indicated concentrations (29). hu-IgG injections were repeated either every third day (1 mg hu-IgG–Fc per animal) or once on day 2 (0.5 mg IVIG, denosumab, or goat IgG). All animals were closely monitored for signs of disease for up to 8 d; gravely moribund mice were euthanized.

Serum preparation

Animals were anesthetized with Isoflurane, and blood was collected by cardiac heart puncture. Blood samples were kept on ice for 30 min and allowed to clot before centrifuging for 10 min at 1700 × *g*, 4˚C. Serum was separated, aliquoted, and frozen immediately at −80˚C until use.

Complement deposition and IgG-binding assays

Bacteria were incubated with increasing amounts (0.1–5%) of normal or hu-C4BP tg mouse serum or indicated IgG preparations for 1 h at 37˚C in 5% CO_2, if not stated explicitly otherwise. For testing the effect of temperature on C4BP binding, we added 150 μg/ml kanamycin, an inhibitor of protein biosynthesis, to all buffers to prevent alterations in the transcriptome due to temperature changes. Bacteria were washed thrice with PBS before and after each staining step. Bacteria were stained to detect surface-bound hu IgG, hu or mouse C4BP, or FH and then analyzed using a CytoFLEX (Beckman Coulter) or a CyFlow Space flow cytometer (Partec).

Polymorphonuclear cell–killing assay

Human polymorphonuclear cells (PMNs) were isolated on a Histopaque and a discontinuous Percoll gradient as described (38). In a 96-well plate, 1 × 10^5 PMNs per well were infected with *S. pyogenes* strain AP1 and AP18 at a multiplicity of infection of 0.1 in the presence of hu-C4BP tg mouse serum and hu-IgG. PMNs were incubated at 37˚C, 5% CO_2 for indicated times. Fifty microliters of the PMN bacteria mixture were diluted serially in PBS, plated onto blood agar, and incubated overnight at 37˚C and 5% CO_2 to enumerate surviving *S. pyogenes*.

Identification of protein H in whole genome sequences

The whole genome raw sequence data for 3615 *S. pyogenes* strains from BioProject PRJNA236767 was downloaded from the NCBI Short Read Archive, consisting of 3615 runs (accession number SRA036051; https://www.ncbi.nlm.nih.gov/sra?term=SRA036051), using the fastq-dump tool from the Short Read Archive Toolkit version 2.5.2 (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software). The raw data were mapped to a reference genome containing the protein H gene (NCTC 8198, accession number LN831034.1) using the high-sensitivity aligner SMALT version 0.7.5. First, the median coverage over the whole genome was determined using genomecov from bedtools version 2.23.0 (39). The coverage of the unique part of protein H (genomic position 1758555–1759380) was determined using SAMtools Mpileup version 0.1.19 (http://www.htslib.org), which recorded the number of bases covered and the average depth of coverage of the whole region. Because protein H amino acid sequences are believed to be hypervariable across strains, an additional search was performed to capture protein sequences that were sufficiently divergent from the reference genome that SMALT was not able to align to them. Using RAPsearch version 2.23 (40) (http://sourceforge.net/projects/smalt/), the data

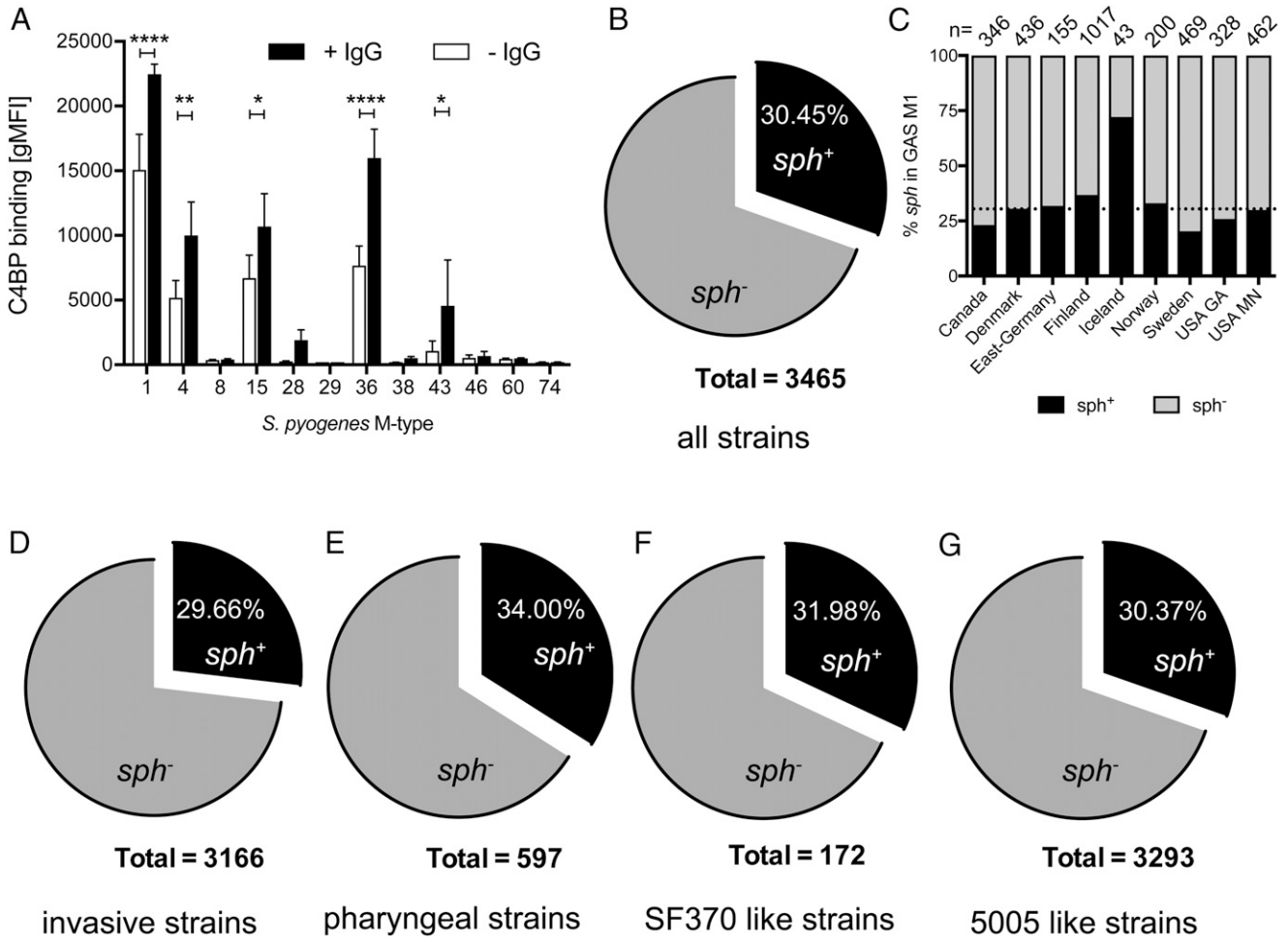

FIGURE 1. C4BP binding in *S. pyogenes strains* and presence of *sph* in M1. Twelve different *S. pyogenes* clinical isolates were tested for their C4BP binding in the presence and absence of hu-IgG (IVIG). Five strains showed significantly increased C4BP binding if coincubated with IgG (**A**). Full genome sequences from 3645 *S. pyogenes* M1 isolates were tested for the presence of *sph*. (**B**) 1055 out of 3645 isolates contain the gene for protein H, *sph*. (**C**) Geospatial analysis revealed that 28.91 ± 5.4% of all isolates from different countries have *sph*. Only Iceland with 72% appears as an outlier, possibly due to low sample numbers. (**D**–**G**) A similar frequency of *sph* was found in invasive strains [(D), 850 out of 2866 isolates] and in pharyngeal isolates [(E), 203 out of 597 isolates]. Interestingly, the distribution of 30% of *sph*$^+$ strains was similar (31.98 and 30.37%, respectively) among SF370 [(F), 55 out of 172 isolates] and 5005-like M1 strains [(G), 1000 out of 3293 isolates], representing older preresurgence strains before 1980 and more contemporary post-resurgence strains, respectively. Our data suggest that protein H has not emerged recently, but likely has persisted in about one-third of all M1 strains for at least the past 50 y. Statistical significance was calculated using a two-way ANOVA with Bonferroni's multiple comparison. Absence of asterisks indicates no significance. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. gMFI, geometric mean fluorescence intensity.

from all samples were mapped against protein sequences of the reference genome, outputting all hits. Every sequence read that had a better bit-score to protein H than to any other protein, and with at least 50% identity, was considered a potential hit.

Size exclusion chromatography

Size exclusion chromatography was performed using an ÄKTAexplorer System (GE Healthcare), employing a Superose 6 10/30 column using PBS as eluent, with 0.6 ml/min flow at ambient temperature. Proteins were incubated together in PBS for 30 min at room temperature before analysis. Nonmixed protein served as controls. Proteins were injected in 200 μl PBS, and the absorbance at 280 nm was recorded to identify the elution profile.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0b. To test for significance, we used one-way or two-way ANOVA analysis with Bonferroni's posttest or Mantel Cox (log-rank; to analyze survival) tests as indicated. A p value < 0.05 was considered to be significant. Sample sizes in animal experiments were chosen to achieve statistical power while minimizing animal use.

Human wound sample

Necrotic tissue was collected from a subject with necrotizing fasciitis (in 2006) in whom *S. pyogenes* M1 was identified as the sole pathogen by the clinical microbiology department of Skåne University Hospital in Lund, Sweden. Although sequence verification of the organism was not performed at that time, the M protein and protein H were identified immunohistochemically.

Study approval

The use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health and the Swedish Animal Welfare Act SFS1988:534. All animal experiments were approved either by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School or by the Laboratory Animal Ethics Committee of Malmö/Lund, Sweden. The ethics committee in Lund approved the use of the human wound material and written informed consent was obtained from human subjects.

Results

hu-IgG augments protein H–C4BP binding

S. pyogenes M proteins are known to bind human serum proteins, especially complement inhibitors and Igs (41–49). In earlier publications, we showed that surface-bound C4BP enhanced virulence of *S. pyogenes* (8, 29). In this study, we sought initially to

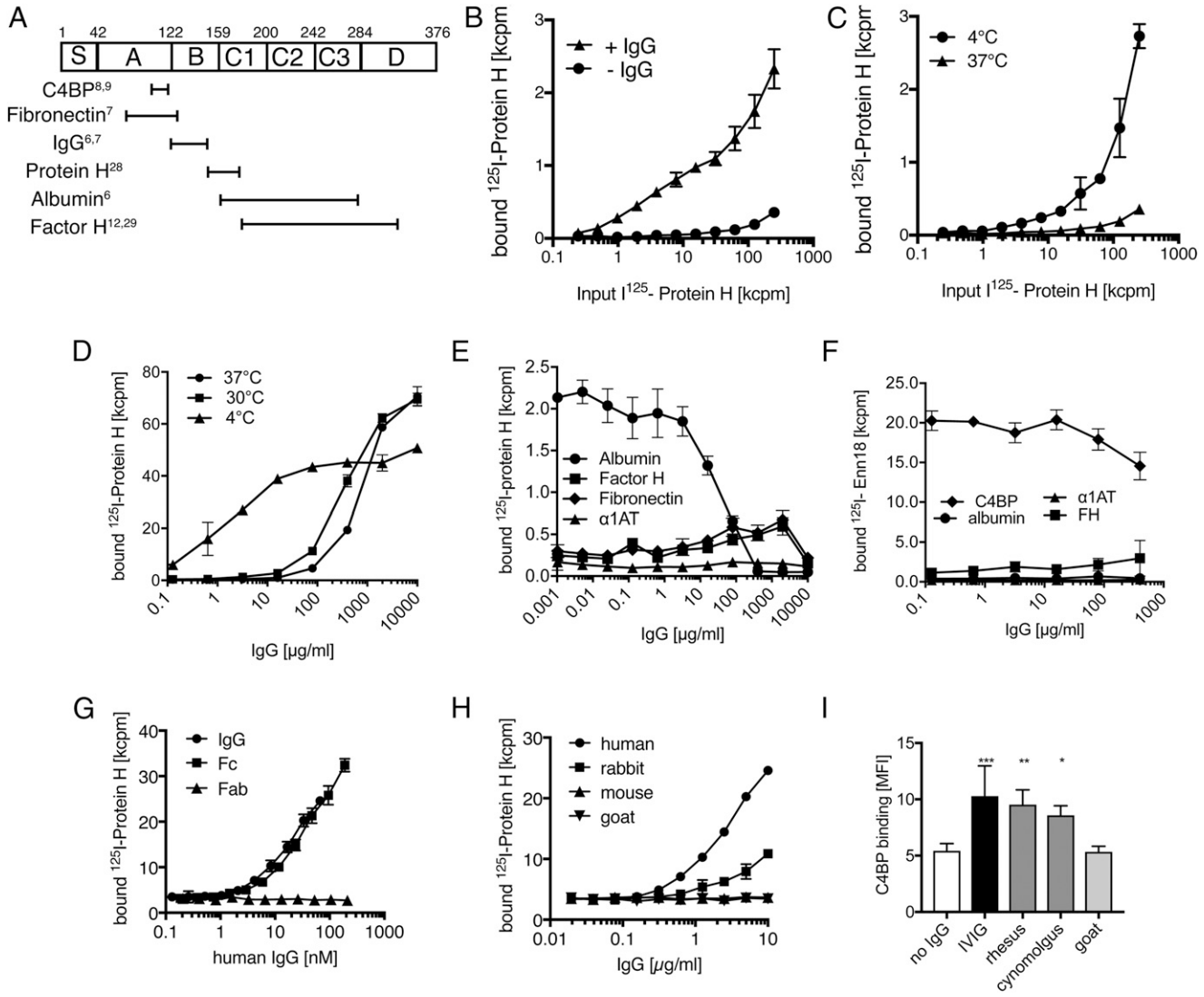

FIGURE 2. Binding of *S. pyogenes* protein H to hu-C4BP: effect of hu-IgG and temperature. (**A**) Schematic representation of protein H and binding sites in protein H for its known ligands (numbers indicate amino acid positions, references in superscript). (**B**) Immobilized C4BP was incubated with increasing amounts of [^{125}I]protein H at 37˚C in the presence or absence of 25 μg/ml IVIG. (**C**) Increasing amounts of [^{125}I]protein H were added to immobilized C4BP at 4 or 37˚C. (**D**) hu-IgG increases [^{125}I]protein H binding to immobilized C4BP at 4, 30, and 37˚C. [^{125}I]protein H (250 kcpm) was incubated with increasing amounts of hu-IgG. (**E**) [^{125}I]protein H (5–270 kcpm) was incubated with increasing amounts of hu-IgG and analyzed for binding to albumin, FH, fibronectin, or α1AT as a negative control. (**F**) [^{125}I]Enn18 was incubated with increasing amounts of hu-IgG and tested for binding to different serum proteins in the presence of different amounts of hu-IgG. (**G**) Immobilized C4BP (1 μg/ml) was incubated with [^{125}I]protein H (125 kcpm) in the presence of increasing amounts of whole hu-IgG, Fc, or Fab fragments. (**H**) Immobilized C4BP was incubated with [^{125}I]protein H (125 kcpm) in the presence of increasing amounts of IgG from either human, rabbit, mouse, or goat. (**I**) *S. pyogenes* AP1 was incubated with 1 mg/ml IgG from the indicated species in 10% mouse serum containing hu-C4BP. Mean (± SD) from three independent determinations are shown in all experiments. Curve comparison for differences was performed using a two-way ANOVA: $p < 0.0001$ (B, F, and H), $p = 0.0001$ (D and E), and $p = 0.002$ (C). MFI = mean fluorescence intensity. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ assessed by one-way ANOVA.

determine the importance of C4BP binding to *S. pyogenes* more generally in the presence of other human serum components, particularly hu-IgG. We tested 12 different *S. pyogenes* isolates that expressed different M proteins (Fig. 1A). Surprisingly, we observed that IgG binding increased C4BP binding in 5 of 12 (42%) M types. All strains that bound C4BP in the absence of IgG bound more C4BP when IgG was present. We chose to characterize the IgG–C4BP interaction on the M1 type strain, AP1 in particular, because this strain is well characterized and is virulent in hu-C4BP tg mice (6–8, 12, 21, 26–29, 35). AP1 binds C4BP and IgG via protein H, a member of the M protein family. We examined 3465 different M1 strains for protein H and found that 30% carried *sph*, the gene that encodes for protein H (Fig. 1B). The frequency of *sph* has been similar in its geospatial distribution (Fig. 1C; except Iceland) and across invasive and pharyngeal isolates (Fig. 1D, 1E) during the past 50 y, here represented by older preresurgence strains before 1980 (Fig. 1F) (50) and more contemporary postresurgence strains (Fig. 1G) (51, 52).

The binding site for C4BP on protein H resides in close proximity to the site where hu-IgG binds (Fig. 2A), suggesting competition between C4BP and IgG binding to protein H (8). Binding of increasing concentrations of ^{125}I-labeled protein H to immobilized C4BP was measured in the presence or absence of 25 μg/ml hu-IgG (IVIG) at 37˚C. IgG enhanced binding of protein H to C4BP at all concentrations of protein H tested. In the absence of IgG, a 100-fold increase in protein H concentration was required before any binding to C4BP was noted (Fig. 2B). Incubation at 4˚C, which induces protein H dimerization (27), increased the

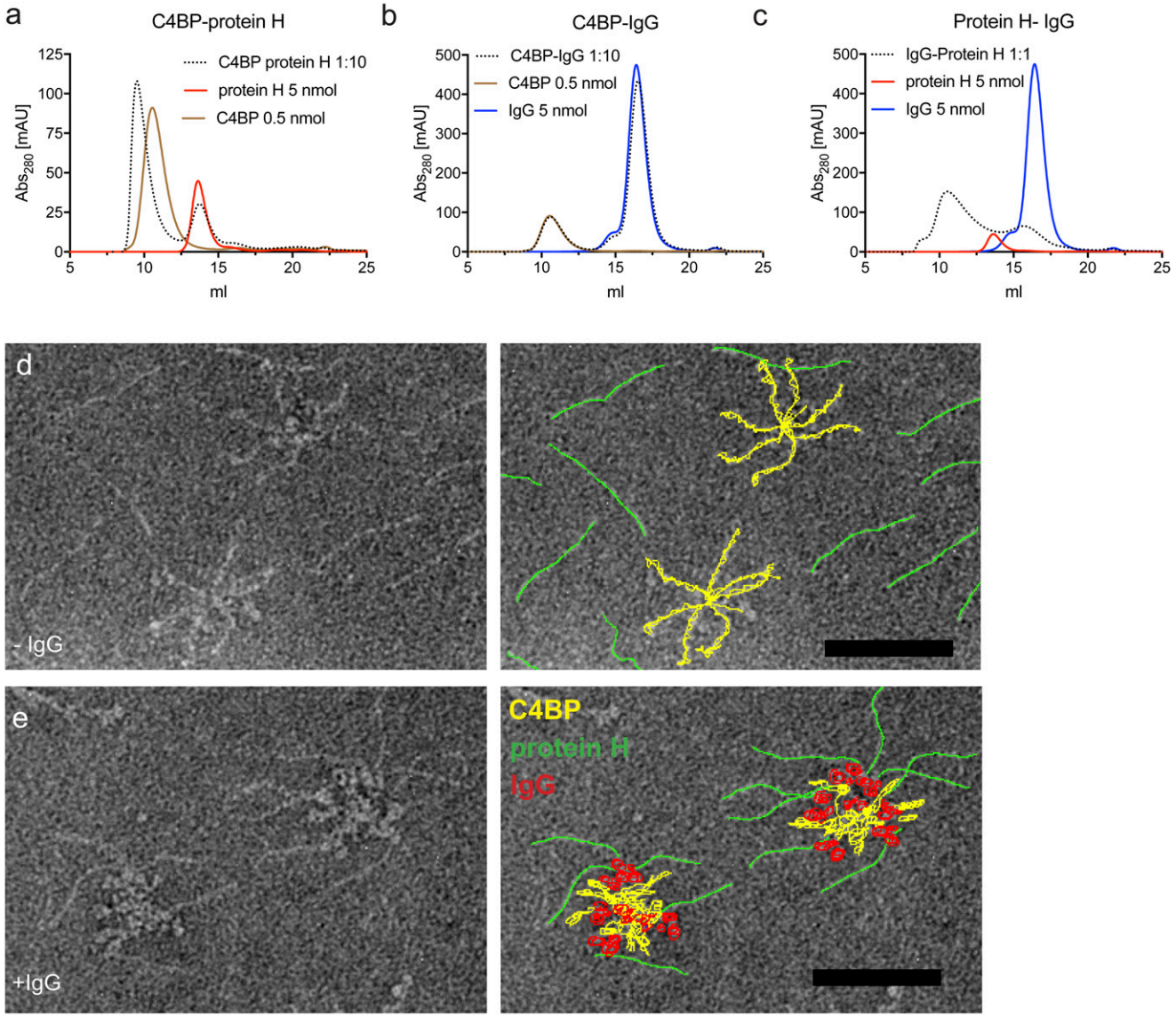

FIGURE 3. Complex formation of C4BP, protein H, and hu-IgG. (**A**) C4BP-protein H, (**B**)C4BP–IgG, and (**C**) protein H–hu-IgG as well as individual proteins were analyzed by size exclusion chromatography on a Superose 6 column. (**D** and **E**) Electron microscopy images of negative-stained protein complexes formed between hu-IgG, protein H, and C4BP. Proteins were artificially colored: C4BP, yellow; protein H, green; and IgG, red. Scale bars, 50 nm. Representative experiments of at least three consistent repetitions are shown.

amount of protein H bound to C4BP at identical protein H concentrations (Fig. 2C). IgG enhanced C4BP–protein H interactions, even at 37˚C (Fig. 2B); therefore, we reasoned that lower temperatures and IgG each increased C4BP binding, possibly by di-/multimerization of protein H.

We next examined the effects of increasing concentrations of IgG on the binding of [^{125}I]protein H to immobilized C4BP at different temperatures (Fig. 2D). IgG enhanced binding of protein H to C4BP at all three temperatures in a (IgG) dose-dependent manner.

Protein H–hu-IgG interaction selectively enhances C4BP binding to protein H

We asked if the protein H–hu-IgG interaction affected the binding of other protein H ligands (Fig. 2A). [^{125}I]protein H was incubated with immobilized albumin, FH, fibronectin, or α1AT in the presence of IgG, except for albumin, which showed a decrease in protein H binding, and no increase in bound concentration to any of the protein H ligands was measured in the presence of increasing concentrations of IgG (Fig. 2E). Thus, hu-IgG specifically, increased the binding of protein H only to C4BP (Fig. 2D, 2E). It is worth noting that the y-axes in Fig 2D and 2E differ; the amount of protein H binding to C4BP at 4˚C, in the presence of 10 μg/ml of IgG, was one to two orders of magnitude greater than binding to other protein H ligands.

We also examined the influence of IgG on the binding of C4BP, FH, albumin, and α1AT to Enn18, an M protein family member of M18 strains (49). [^{125}I]Enn18 binding to C4BP did not increase because of IgG, whereas no binding to FH, albumin, or α1AT was observed (Fig. 2F). Taken together, IgG interacts with protein H to specifically increase the amount of C4BP that is bound but does not increase affinity for any of the other ligands tested.

To identify the IgG region containing the binding sites for protein H, we incubated [^{125}I]protein H with immobilized C4BP in the presence of increasing amounts of intact IgG or corresponding concentrations of Fc or Fab fragments (Fig. 2G). Complex formation resided exclusively in hu-Fc–containing fragments. Non–hu-IgG did not increase the affinity of protein H for hu-C4BP (or to a low extent in the case of rabbit IgG; Fig. 2H). Similar to hu-IgG, nonhuman primate IgG increased the amount of C4BP that bound to AP1 (Fig. 2I).

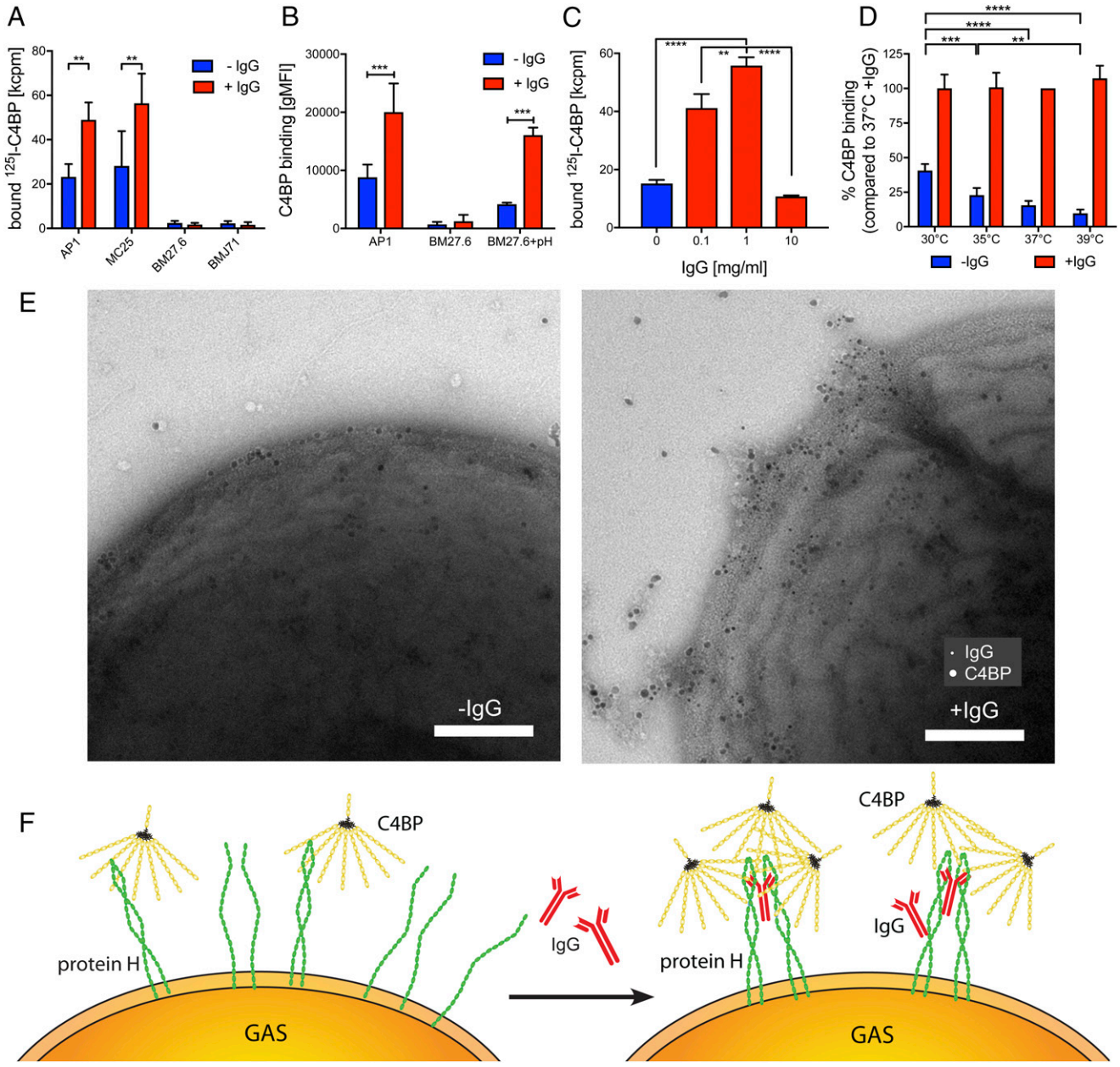

FIGURE 4. Hu-IgG increases C4BP-binding to bacteria. (**A**) *S. pyogenes* strains AP1 and isogenic mutants MC25 (protein H$^-$), BM27.6 (protein M$^-$), and BMJ71 (protein M$^-$/H$^-$) were incubated with [^{125}I]C4BP (100 kcpm) in the presence or absence of 1 mg/ml hu-IgG. (**B**) AP1, BM27.6, and protein H–complemented mutant BM27.6 + pH were incubated in 10% mouse serum containing hu-C4BP in the presence or absence of 1 mg/ml hu-IgG. (**C**) AP1 was incubated with [^{125}I]C4BP (100 kcpm) and increasing amounts of hu-IgG at 37°C. (**D**) AP1 was incubated within 10% mouse serum containing hu-C4BP in the presence or absence of 1 mg/ml hu-IgG at the indicated temperatures. (**E**) Electron microscopic confirmation of increased C4BP binding to AP1 in the presence of hu-IgG. (**F**) Hypothetical model of IgG-induced binding of C4BP to protein H. Mean (± SD) from three independent determinations are shown. More than 500 interactions from different areas of the microscopy grids were analyzed. Scale bars, 100 nm. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ assessed by one-way (C) or two-way (A, B, and D) ANOVA.

hu-IgG forms complexes with protein H and C4BP

To verify the observed protein–protein interaction, we performed size exclusion chromatography of C4BP–protein H, C4BP–IgG, and protein H–IgG complexes. Compared to the individual proteins, C4BP and protein H formed a complex with a corresponding reduction in the free protein H peak, judged by the different elution volumes (Fig. 3A). In contrast, C4BP and IgG did not interact (Fig. 3B). Protein H–IgG bound each other, resulting in a new peak (Fig. 3C).

Furthermore, we visualized complex formation between the three proteins using electron microscopy. Protein H was incubated with C4BP either in the absence (Fig. 3D) or presence (Fig. 3E) of IgG. In the absence of IgG, we found only ~7% of C4BP molecules (yellow) complexed with protein H (green). In the presence of IgG (red), 83% of C4BP molecules were complexed with protein H.

S. pyogenes *binds larger amounts of C4BP in the presence of IgG*

We incubated AP1 and its isogenic mutant strains MC25, BM27.6, and BJM71 with ^{125}I-C4BP in the presence or absence of IgG (Fig. 4A). AP1 and its isogenic mutant MC25 (both expressing protein H) bound C4BP, which increased significantly in the presence of IgG. Mutant strains BM27.6 and BMJ71, lacking

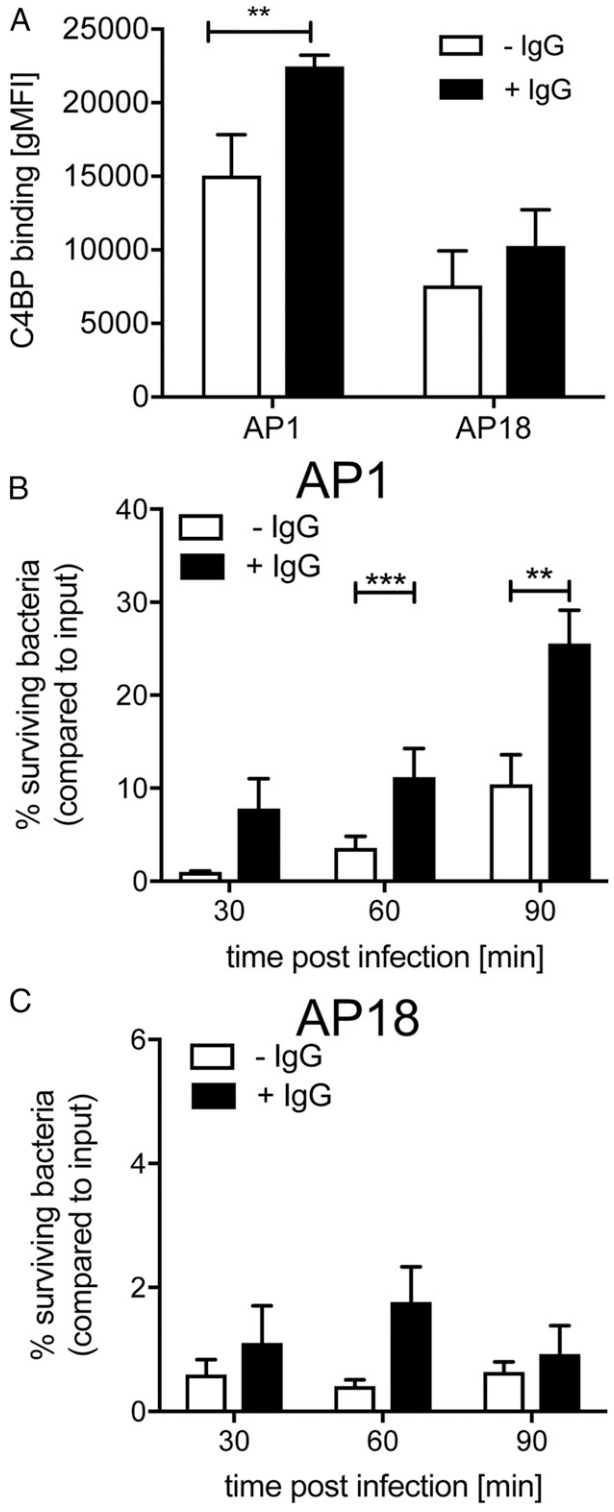

FIGURE 5. Hu-IgG enhances C4BP binding to bacteria and promotes bacterial survival. To assess complement deposition, different *S. pyogenes* strains were incubated in hu-C4BP tg mouse serum and analyzed for C4BP binding (**A**) in the presence and absence of hu-IgG. Survival assay of *S. pyogenes* AP1 (**B**) and AP18 (**C**) coincubated with isolated PMNs and hu-C4BP tg mouse serum in the presence and absence of hu-IgG. Mean (± SD) from three independent determinations are shown. Statistical significance was calculated using a two-way ANOVA with Bonferroni's multiple comparison. Absence of asterisks indicates no significance. $^{**}p < 0.01$, $^{***}p < 0.001$. gMFI, geometric mean fluorescence intensity.

protein H, did not bind C4BP independent of the presence of IgG. Complementing protein H in BM27.6 (BM27.6 + pH) restored increased C4BP binding due to IgG (Fig. 4B).

We analyzed the influence of different IgG concentrations on C4BP binding to *S. pyogenes* AP1. C4BP binding to AP1 was maximal in the presence of 1 mg/ml of IgG (Fig. 4C). At 10 mg/ml IgG, C4BP levels decreased to levels similar to that seen in the absence of IgG.

C4BP binding to intact bacteria in the presence of IgG was not affected by temperature (Fig. 4D). In the absence of IgG, however, we found that increasing temperature significantly decreased C4BP binding capacity of AP1 from 40% at 30°C to <10% at 39°C, compared with binding of C4BP to bacteria in the presence of IgG across these temperatures.

Using gold-labeled C4BP (10 nm) and IgG (5 nm), we visualized the binding of C4BP to AP1 by electron microscopy (Fig. 4E). In the presence of IgG, we noted more C4BP (917 ± 84 gold particles/mm^2) bound to the surface than without IgG (82 ± 8 gold particles/mm^2). The majority of bound C4BP and IgG were in close proximity. No binding to bacteria was observed with control polyethylene glycol–coated gold particles of the same size (Supplemental Fig. 1A–C). We hypothesize that protein H di/multimerizes in the presence of hu-IgG, which permits greater binding to C4BP (Fig. 4F).

Hu-IgG increases the amount of C4BP that binds to bacterial surface proteins and prevents opsonophagocytic killing

We compared C4BP binding to *S. pyogenes* AP1 and AP18 in the presence of IgG. Only AP1, but not AP18, showed a significant increase in bound C4BP consistent with the observed effect of IgG to purified protein H and Enn18 (Fig. 5A).

Next, we assessed the influence of IgG on the killing of *S. pyogenes* by PMNs. Addition of IgG to AP1 significantly increased survival at 60 and 90 min (Fig. 5B) but did not alter survival of AP18 (Fig. 5C).

Taken together, these data show that hu-IgG increases the amount of C4BP that binds to surface AP1, but not AP18, of *S. pyogenes*, which reduces opsonophagocytic killing.

Enhancement of C4BP binding to S. pyogenes *by hu-IgG increases lethality in mice*

AP1 does not bind mouse C4BP or FH (Supplemental Fig. 2A–B). In contrast, in hu-C4BPxFH tg mouse serum, binding of hu-C4BP was augmented ~2-fold upon adding 1 mg/ml hu-IgG to the mouse serum (Supplemental Fig. 2C), whereas binding of hu-FH in tg mouse serum was not increased upon hu-IgG addition (Supplemental Fig. 2D). Interestingly, analysis of binding curves (Supplemental Fig. 2C) revealed that adding IgG does not increase the affinity (K_D) of C4BP for AP1, compared with analysis when IgG is absent. However, maximal binding of C4BP to AP1 nearly doubled when IgG was present, indicating more C4BP binding sites on the bacteria in the presence of IgG.

To exclude the effect of Fab-directed opsonization in vivo, we used IgG-Fc fragments. Hu-C4BP tg mice were treated 1 d prior to infection (Day −1), either with 1 mg IgG-Fc or mock treated as a control. All infected animals treated with IgG Fc succumbed to infection by Day 4 (Fig. 6A). Mock-treated and infected animals survived significantly longer. Injection of 1 mg of Fc on Days −1, 2, and 5 yielded peak serum concentrations of up to 1.8 μM (Supplemental Fig. 3A). Fc increased C4BP binding to AP1, similarly to whole hu-IgG (Fig. 6B).

To determine if intact hu-IgG has similar effects on C4BP binding to *S. pyogenes* AP1, we used an unrelated hu-IgG2 mAb, denosumab. Neither denosumab nor goat IgG opsonized AP1

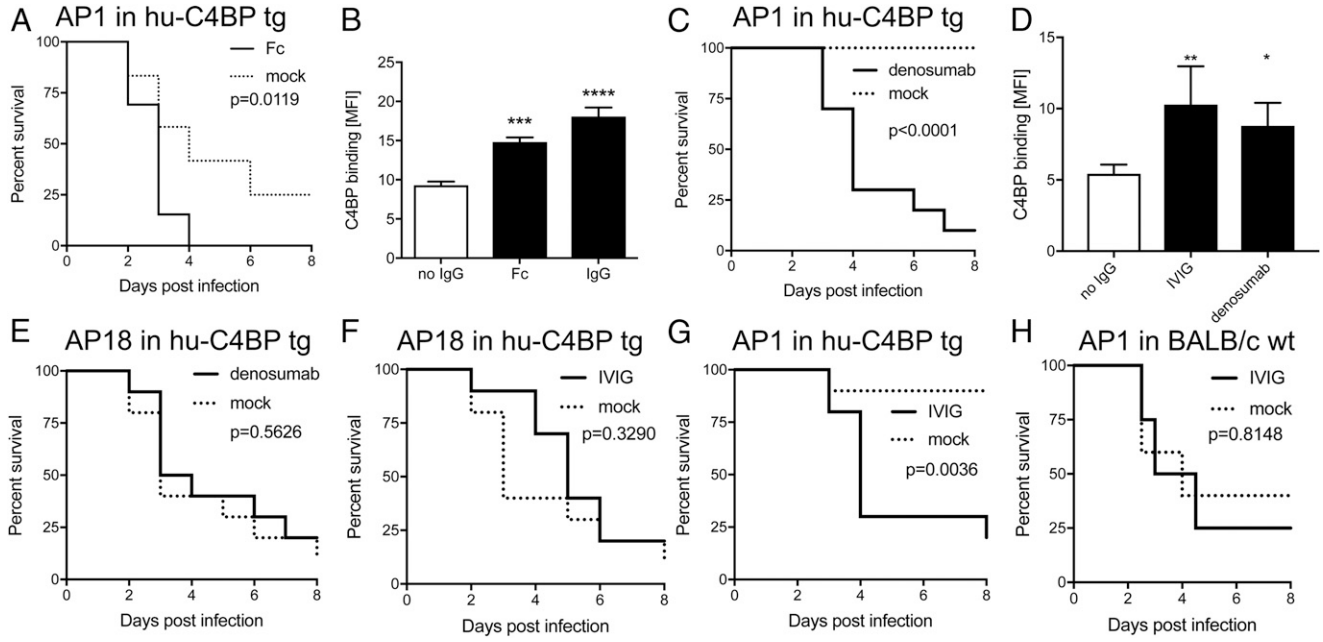

FIGURE 6. Hu-IgG increases lethality of *S. pyogenes* infection in hu-C4BP tg mice. (**A**) Hu-C4BP tg animals were injected i.p. with either 1 mg hu-IgG–Fc ($n = 13$) or mock treated ($n = 12$) 24 h prior to i.v. infection with 1.5×10^7 *S. pyogenes* AP1. (**B**) hu-IgG–Fc fragments exhibit similar effects as intact hu-IgG (IVIG) on C4BP binding to *S. pyogenes*. (**C** and **E–H**) BALB/c or hu-C4BP tg animals were injected i.p. with either 2 mg denosumab, IVIG, or mock treated 24 h prior to i.v. infection with *S. pyogenes* M18 or AP1 and monitored for 8 d. (C) Hu-C4BP animals ($n = 10$ per group) were treated with denosumab or mock treated, then infected with 1.25×10^7 *S. pyogenes* AP1. (**D**) C4BP binding to *S. pyogenes* in the presence of equivalent amounts of IVIG or denosumab. (E and F) Hu-C4BP tg mice ($n = 10$ per group) were given denosumab (E), IVIG (F), or mock treated, then infected with 4×10^7 *S. pyogenes* AP18. (G) Hu-C4BP tg mice ($n = 10$ per group) were given IVIG or mock treated, then infected with 2.5×10^7 *S. pyogenes* AP1. (H) BALB/c animals (IVIG $n = 4$ and PBS $n = 5$) were given IVIG or mock treated, then infected with 3×10^7 *S. pyogenes* AP1. Mean (± SD) from three independent experiments are shown (B and D). Control groups in (A) and (H) received PBS, and controls in (C) and (E)–(G) received goat IgG. Statistical significance was calculated using Mantel-Cox analysis (A, C, and E–H) and one-way ANOVA (B and D); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. MFI, mean fluorescence intensity.

(Supplemental Fig. 3C). We pretreated hu-C4BP tg animals with 2 mg denosumab per animal on Day −1, to achieve serum hu-IgG levels of 6.6 μM (1 mg/ml). On Day 2, we reinjected 0.5 mg of denosumab, which led to peak serum levels of up to 9.0 μM (Supplemental Fig. 3B). Ninety percent of the animals treated with denosumab succumbed to infection, whereas all mock-treated animals survived (Fig. 6C). Similar results were achieved using a different AP1 inoculum (Supplemental Fig. 3D). Denosumab increased C4BP binding to AP1 in vitro (Fig. 6D), similar to hu-IgG, supporting the role of hu-IgG in enhancing AP1 infection.

To underline the importance of C4BP's interaction with protein H in IgG-mediated virulence, we used *S. pyogenes* AP18, in which C4BP binding is not affected by hu-IgG (Fig. 5A). Administration of denosumab did not change mortality in AP18-infected mice compared with mock-treated animals (Fig. 6E). Similar to denosumab, AP18-infected hu-C4BP animals treated with hu-IgG (IVIG) showed no significant difference in survival compared with mock-treated animals (Fig. 6F). In contrast, IgG treatment of tg hu-C4BP mice prior to infection with AP1 significantly decreased their survival (Fig. 6G), similar to IgG-Fc (Fig. 6A) and denosumab (Fig. 6C).

Hu-IgG treatment of BALB/c WT mice did not affect survival compared with mock treatment during infection with AP1 (Fig. 6H, Supplemental Fig. 3E). Similarly, denosumab did not increase mortality of AP1-infected animals (Supplemental Fig. 3F) because *S. pyogenes* cannot recruit and use mouse C4BP. Goat IgG did not influence C4BP binding to bacteria (Fig. 1I) and thus did not alter the course of infection in mice compared with PBS treatment (Supplemental Fig. 3G).

Taken together, these data show a detrimental effect of hu-IgG on *S. pyogenes* infection in tg hu-C4BP–expressing mice.

IgG increases C4BP binding to S. pyogenes *M1 in humans*

We sought to verify the interaction of C4BP, IgG, and protein H in humans by analyzing tissue samples from a subject with necrotizing fasciitis caused solely by *S. pyogenes* that expressed M1 protein. Scanning and transmission electron micrographs from biopsy samples confirmed the presence of bacteria, indicated by white (Fig. 7A) and black (Fig. 7B) arrows. We stained these samples for C4BP, protein H, and IgG or M protein (Fig. 7C–G, Supplemental Fig. 1D–G). Because protein H expression is regulated during the course of infection, we found examples of *S. pyogenes* M1 with or without protein H in the same sample. Protein H–positive M1 bacteria bound >3.5 times more anti-C4BP–labeled gold particles than protein H–negative bacteria (273 ± 38 C4BP particles/μm^2 versus 73 ± 21 C4BP particles/μm^2; Fig. 7E). Colocalization analysis revealed that C4BP colocalized 79 ± 7% of the time with protein H and IgG (Fig. 7F, 7G), whereas it localized with protein H alone in only 32 ± 5% of instances in the absence of IgG (Fig. 7H). These data provide evidence that IgG enhances C4BP binding to *S. pyogenes* during human infection.

Discussion

We have identified a novel virulence mechanism of *S. pyogenes*, namely that hu-IgG increases the amount of C4BP that binds to *S. pyogenes* protein H. Binding of IgG leads to dimerization of protein H on the bacterial surface (27). Clustered protein H is then able to bind larger amounts of C4BP than monomeric protein H.

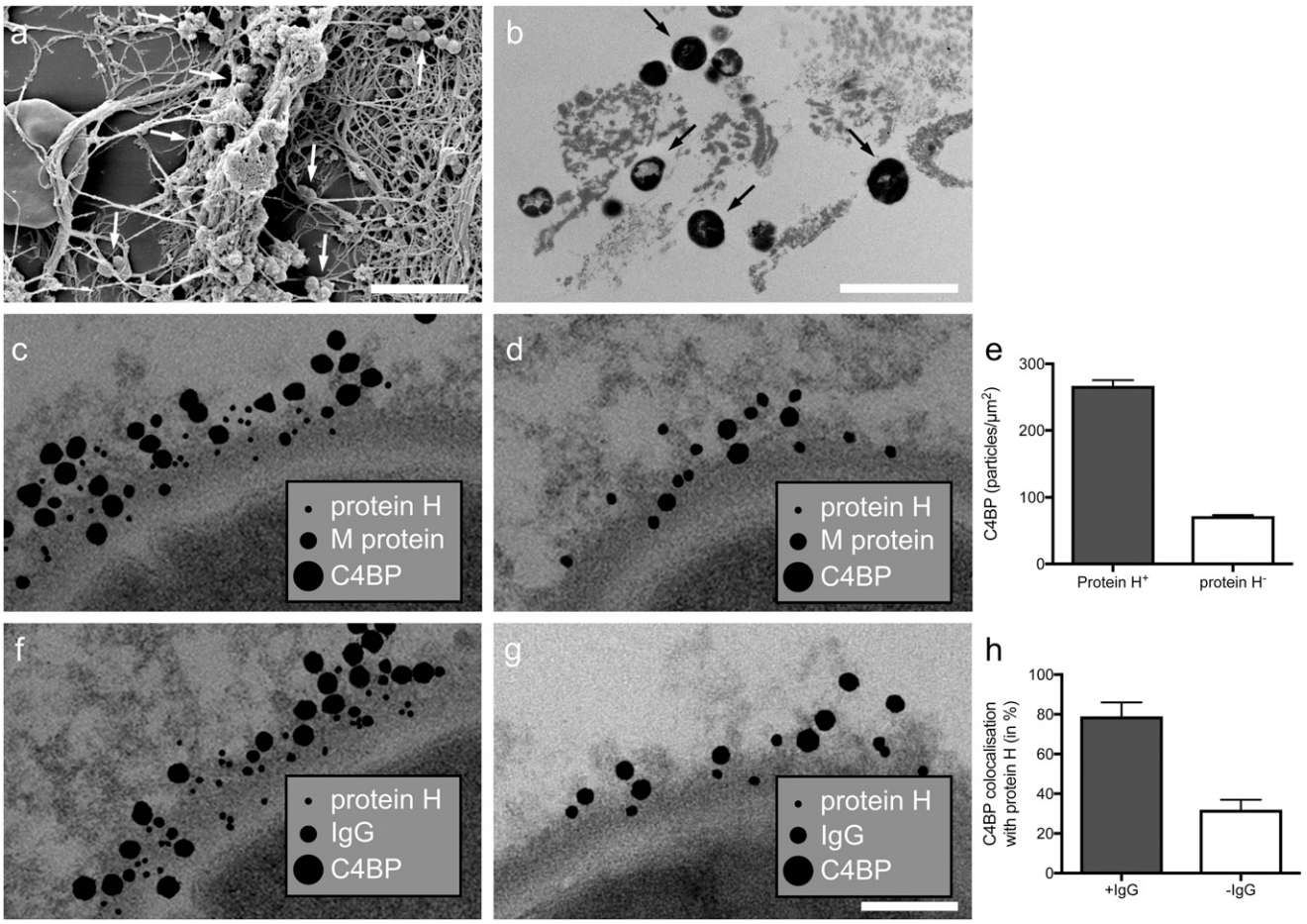

FIGURE 7. Colocalization of protein H, IgG, and C4BP in tissue samples from a patient with necrotizing fasciitis. (**A**) Scanning electron microscopy of surgically excised necrotic tissue from a subject with necrotizing fasciitis of the left shoulder. White arrows indicate streptococci in the tissue. (**B**) Transmission electron micrograph from the same sample shows bacteria (black arrows), which are subsequently stained with different Abs. (**C**, **D**, **F**, and **G**) Bacteria are stained either with Abs coupled to gold particles (size in brackets) that react with C4BP (15 nm), (C and D) M protein (10 nm) or (F and G) IgG (10 nm), and protein H (5 nm). (C) *S. pyogenes* M1 that express protein H showed more C4BP (273 ± 38 gold particles/μm^2) compared with protein H–negative *S. pyogenes* M1 bacteria [(D), 73 ± 21 gold particles/μm^2], quantified in (**E**). IgG colocalizes with C4BP in protein H–positive bacteria (F) but to a lesser extent on protein H–negative bacteria (G). (**H**) C4BP–protein H colocalization is increased in the presence of IgG (79 ± 7%) compared with the absence of IgG (32 ± 5%). Scale bars, 5 μm (A), 2 μm (B), or 100 nm (C–F). Mean (± SD) of more than 50 bacteria are shown (E and H).

Thereafter, bound C4BP limits complement activation and reduces opsonization and bacterial elimination by phagocytes. IgG not only increases the amount of C4BP that binds to purified protein H in vitro, but it also increases binding of C4BP to bacteria in vivo, thereby reducing complement activation and opsonophagocytosis. We found that hu-IgG enhances *S. pyogenes* infection in mice. Consistent with our findings, we also colocalized IgG, C4BP, and protein H in human tissues from necrotizing fasciitis caused by *S. pyogenes*. This virulence mechanism may be particularly important in niches such as mucosal surfaces and interstitial fluids where the availability of C4BP is diminished. Furthermore, we have evidence that this mechanism is not limited to M1 strains that express protein H but also occurs in other M type strains, thereby extending and strengthening the significance of this observation. All *S. pyogenes* strains that we tested that bound C4BP in the absence of IgG showed significant increased C4BP binding in the presence of IgG.

Protein H belongs to the family of M proteins and is expressed exclusively in M1 strains of *S. pyogenes*, the most frequently expressed M protein serotype in the Western world and the main cause of invasive and often lethal streptococcal infections (24, 53) (including 1, 14, 24, 54–59). Protein H is expressed in 28% (18 out of 64) of M1 serotype that cause necrotizing fasciitis, according to an analysis (60) of isolates from a CDC surveillance study (53). We confirmed that 30% (1055/3465) of *S. pyogenes* M1 strains had the *sph* gene encoding protein H or a homolog (51).

Because low temperatures induce dimerization of protein H (27, 61) and because IgG also exhibited a similar effect on protein H–C4BP binding, we speculate that IgG also di-/polymerizes protein H. This synergistic effect was supported by electron microscopy showing that IgG increased the prevalence of C4BP–protein H complex.

Analysis of monomeric recombinant protein H revealed no measurable affinity for their putative ligands (27). Dimerization of protein H, induced by lower temperatures or by binding of hu-IgG Fc, permitted C4BP binding. However, on the bacterial surface, we found no effect of IgG on affinity for C4BP binding to protein H at any IgG concentration. Of note, maximal binding increased ~2-fold at higher temperatures (e.g., 37°C) in the presence of IgG. As a consequence, protein H dimerized, creating more binding sites for C4BP. We propose that protein H–protein H dimers are stabilized under these conditions, thus allowing for greater binding of C4BP to protein H.

We demonstrated that the Fc region in hu-IgG increased C4BP–protein H interaction and that rabbit IgG also bound to protein H. This finding is consistent with previous observations that protein

H binds to rabbit, baboon, and guinea pig IgG but not to rat, mouse, bovine, or equine IgG (21, 35). Orientation of IgG on the surface of *S. pyogenes* depends on IgG concentrations. Extravascular fluids for example have lower levels of C4BP and less IgG compared with serum (62). The concentration of C4BP bound to *S. pyogenes* was maximal at 1 mg/ml IgG (~1/5th–1/10th of serum concentration in healthy adults; see Ref. 63), consistent with our assertion that *S. pyogenes* has adapted to bind C4BP in environments with low levels of both C4BP and hu-IgG, such as extravascular compartments. In IgG-poor environments, *S. pyogenes* binds IgG via the Fc region predominantly; in undiluted serum, the interaction is mediated mainly by specific IgG Abs binding to their antigenic targets on the streptococcal surface via Fab2 (41). *S. pyogenes* counteracts Ig-mediated opsonization by secreting enzymes that cleave and inactivate surface-bound IgG (2, 64, 65). It is believed that the binding of IgG Abs via effector Fc fragments to microbes renders them immunologically effete (66), e.g., by preventing Fc-receptors from recognizing IgG-opsonized bacteria (48). In this study, we provide evidence that IgG binding is indeed an immune evasion mechanism. C4BP and IgG share a common binding site at the C terminus of domain A and the N terminus of domain B (6, 8), and this could explain why we were unable to identify any strains among the 12 tested that bound to only one of these proteins.

C4BP bound to *S. pyogenes* increases adherence and invasion of endothelial cells (8). Thus, enhancement of C4BP binding to protein H in the presence of hu-IgG may facilitate bacterial invasion. The virulence mechanism described in this article explains how IgG enables *S. pyogenes* to bind C4BP at temperatures encountered in vivo; protein H alone does not recruit C4BP at these temperatures. However, SpeB, a cysteine protease secreted by *S. pyogenes*, cleaves protein H to release a 36 kDa fragment, which contains the IgG binding region (67). Recruitment of C4BP to the released protein H–IgG complex could further enlarge these immune complexes rapidly and contribute to microthrombus formation, glomerulonephritis, and acute renal failure, all of which are complications of invasive streptococcal infections (68, 69).

These data highlight a novel mechanism that certain strains of *S. pyogenes* may use to flourish in their natural ecological niches, the throat and skin, where lower levels of IgG and complement proteins exist. The results also emphasize the significance of complement inhibition in the pathogenicity of *S. pyogenes* infection, a phenomenon originally described for FH (5). Targeting interactions between bacteria and host complement inhibitors may offer new opportunities to treat invasive *S. pyogenes* infection.

Acknowledgments

We thank Oonagh Shannon (Lund University) and Nancy Nowak and Bo Zhang (both of University of Massachusetts Medical School) for expert help with breeding and caring for mice, Maria Baumgarten for her skillful technical assistance, and the Core Facility for Integrated Microscopy at the Panum Institute, Copenhagen University for help with electron microscopy.

Disclosures

The authors have no financial conflicts of interest.

References

1. Carapetis, J. R., A. C. Steer, E. K. Mulholland, and M. Weber. 2005. The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* 5: 685–694.
2. Cunningham, M. W. 2000. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* 13: 470–511.
3. Bisno, A. L., and D. L. Stevens. 1996. Streptococcal infections of skin and soft tissues. *N. Engl. J. Med.* 334: 240–245.
4. Nowak, R. 1994. Flesh-eating bacteria: not new, but still worrisome. *Science* 264: 1665.
5. Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti. 1988. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc. Natl. Acad. Sci. USA* 85: 1657–1661.
6. Frick, I. M., P. Akesson, J. Cooney, U. Sjöbring, K. H. Schmidt, H. Gomi, S. Hattori, C. Tagawa, F. Kishimoto, and L. Björck. 1994. Protein H–a surface protein of *Streptococcus pyogenes* with separate binding sites for IgG and albumin. *Mol. Microbiol.* 12: 143–151.
7. Frick, I. M., K. L. Crossin, G. M. Edelman, and L. Björck. 1995. Protein H–a bacterial surface protein with affinity for both immunoglobulin and fibronectin type III domains. *EMBO J.* 14: 1674–1679.
8. Ermert, D., A. Weckel, V. Agarwal, I. M. Frick, L. Björck, and A. M. Blom. 2013. Binding of complement inhibitor C4b-binding protein to a highly virulent *Streptococcus pyogenes* M1 strain is mediated by protein H and enhances adhesion to and invasion of endothelial cells. *J. Biol. Chem.* 288: 32172–32183.
9. Thern, A., L. Stenberg, B. Dahlbäck, and G. Lindahl. 1995. Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. *J. Immunol.* 154: 375–386.
10. Carlsson, F., C. Sandin, and G. Lindahl. 2005. Human fibrinogen bound to *Streptococcus pyogenes* M protein inhibits complement deposition via the classical pathway. *Mol. Microbiol.* 56: 28–39.
11. Ly, D., J. M. Taylor, J. A. Tsatsaronis, M. M. Monteleone, A. S. Skora, C. A. Donald, T. Maddocks, V. Nizet, N. P. West, M. Ranson, et al. 2014. Plasmin(ogen) acquisition by group A *Streptococcus* protects against C3b-mediated neutrophil killing. *J. Innate Immun.* 6: 240–250.
12. Blom, A. M., M. Magda, L. Kohl, J. Shaughnessy, J. D. Lambris, S. Ram, and D. Ermert. 2017. Factor H-IgG chimeric proteins as a therapeutic approach against the gram-positive bacterial pathogen *Streptococcus pyogenes. J. Immunol.* 199: 3828–3839.
13. LaRock, C. N., S. Döhrmann, J. Todd, R. Corriden, J. Olson, T. Johannssen, B. Lepenies, R. L. Gallo, P. Ghosh, and V. Nizet. 2015. Group A streptococcal M1 protein sequesters cathelicidin to evade innate immune killing. *Cell Host Microbe* 18: 471–477.
14. Walker, M. J., T. C. Barnett, J. D. McArthur, J. N. Cole, C. M. Gillen, A. Henningham, K. S. Sriprakash, M. L. Sanderson-Smith, and V. Nizet. 2014. Disease manifestations and pathogenic mechanisms of group A *Streptococcus. Clin. Microbiol. Rev.* 27: 264–301.
15. Barnett, T. C., D. Liebl, L. M. Seymour, C. M. Gillen, J. Y. Lim, C. N. Larock, M. R. Davies, B. L. Schulz, V. Nizet, R. D. Teasdale, and M. J. Walker. 2013. The globally disseminated M1T1 clone of group A *Streptococcus* evades autophagy for intracellular replication. *Cell Host Microbe* 14: 675–682.
16. Honda-Ogawa, M., T. Ogawa, Y. Terao, T. Sumitomo, M. Nakata, K. Ikebe, Y. Maeda, and S. Kawabata. 2013. Cysteine proteinase from *Streptococcus pyogenes* enables evasion of innate immunity via degradation of complement factors. *J. Biol. Chem.* 288: 15854–15864.
17. Uchiyama, S., F. Andreoni, R. A. Schuepbach, V. Nizet, and A. S. Zinkernagel. 2012. DNase Sda1 allows invasive M1T1 Group A *Streptococcus* to prevent TLR9-dependent recognition. *PLoS Pathog.* 8: e1002736.
18. Walport, M. J. 2001. Complement. First of two parts. *N. Engl. J. Med.* 344: 1058–1066.
19. Zipfel, P. F., T. Hallström, and K. Riesbeck. 2013. Human complement control and complement evasion by pathogenic microbes–tipping the balance. *Mol. Immunol.* 56: 152–160.
20. Ermert, D., and A. M. Blom. 2016. C4b-binding protein: the good, the bad and the deadly. Novel functions of an old friend. *Immunol. Lett.* 169: 82–92.
21. Akesson, P., J. Cooney, F. Kishimoto, and L. Björck. 1990. Protein H–a novel IgG binding bacterial protein. *Mol. Immunol.* 27: 523–531.
22. Fischetti, V. A. 2016. M protein and other surface proteins on streptococci. In *Streptococcus Pyogenes: Basic Biology to Clinical Manifestations.* J. J. Ferretti, D. L. Stevens, and V. A. Fischetti, eds. University of Oklahoma Health Sciences Center, Oklahoma City, OK.
23. Sanderson-Smith, M., D. M. De Oliveira, J. Guglielmini, D. J. McMillan, T. Vu, J. K. Holien, A. Henningham, A. C. Steer, D. E. Bessen, J. B. Dale, et al; M Protein Study Group. 2014. A systematic and functional classification of *Streptococcus pyogenes* that serves as a new tool for molecular typing and vaccine development. *J. Infect. Dis.* 210: 1325–1338.
24. Steer, A. C., I. Law, L. Matatolu, B. W. Beall, and J. R. Carapetis. 2009. Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. *Lancet Infect. Dis.* 9: 611–616.
25. Berge, A., B. M. Kihlberg, A. G. Sjöholm, and L. Björck. 1997. Streptococcal protein H forms soluble complement-activating complexes with IgG, but inhibits complement activation by IgG-coated targets. *J. Biol. Chem.* 272: 20774–20781.
26. Kihlberg, B. M., M. Collin, A. Olsén, and L. Björck. 1999. Protein H, an antiphagocytic surface protein in *Streptococcus pyogenes. Infect. Immun.* 67: 1708–1714.
27. Akerström, B., G. Lindahl, L. Björck, and A. Lindqvist. 1992. Protein Arp and protein H from group A streptococci. Ig binding and dimerization are regulated by temperature. *J. Immunol.* 148: 3238–3243.
28. Frick, I. M., M. Mörgelin, and L. Björck. 2000. Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein-protein interactions. *Mol. Microbiol.* 37: 1232–1247.
29. Ermert, D., J. Shaughnessy, T. Joeris, J. Kaplan, C. J. Pang, E. A. Kurt-Jones, P. A. Rice, S. Ram, and A. M. Blom. 2015. Virulence of group A streptococci is enhanced by human complement inhibitors. *PLoS Pathog.* 11: e1005043.
30. Collin, M., and A. Olsén. 2000. Generation of a mature streptococcal cysteine proteinase is dependent on cell wall-anchored M1 protein. *Mol. Microbiol.* 36: 1306–1318.
31. Kihlberg, B. M. 1998. Immunoglobulin-binding bacterial surface proteins: biomedical tools and virulence factors. In *Dept. of Cell and Molecular Biology.* Lund University, Lund, Sweden, p. 146.

32. Kihlberg, B. M., J. Cooney, M. G. Caparon, A. Olsén, and L. Björck. 1995. Biological properties of a *Streptococcus pyogenes* mutant generated by Tn916 insertion in mga. *Microb. Pathog.* 19: 299–315.
33. Härdig, Y., A. Hillarp, and B. Dahlbäck. 1997. The amino-terminal module of the C4b-binding protein alpha-chain is crucial for C4b binding and factor I-cofactor function. *Biochem. J.* 323: 469–475.
34. Sim, E., M. S. Palmer, M. Puklavec, and R. B. Sim. 1983. Monoclonal antibodies against the complement control protein factor H (beta 1 H). *Biosci. Rep.* 3: 1119–1131.
35. Akesson, P., K. H. Schmidt, J. Cooney, and L. Björck. 1994. M1 protein and protein H: IgGFc- and albumin-binding streptococcal surface proteins encoded by adjacent genes. *Biochem. J.* 300: 877–886.
36. Engel, J., and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI, and proteoglycans. *Methods Enzymol.* 145: 3–78.
37. Kostenuik, P. J., H. Q. Nguyen, J. McCabe, K. S. Warmington, C. Kurahara, N. Sun, C. Chen, L. Li, R. C. Cattley, G. Van, et al. 2009. Denosumab, a fully human monoclonal antibody to RANKL, inhibits bone resorption and increases BMD in knock-in mice that express chimeric (murine/human) RANKL. *J. Bone Miner. Res.* 24: 182–195.
38. Ermert, D., A. Zychlinsky, and C. Urban. 2009. Fungal and bacterial killing by neutrophils. *Methods Mol. Biol.* 470: 293–312.
39. Quinlan, A. R., and I. M. Hall. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26: 841–842.
40. Zhao, Y., H. Tang, and Y. Ye. 2012. RAPSearch2: a fast and memory-efficient protein similarity search tool for next-generation sequencing data. *Bioinformatics* 28: 125–126.
41. Nordenfelt, P., S. Waldemarson, A. Linder, M. Mörgelin, C. Karlsson, J. Malmström, and L. Björck. 2012. Antibody orientation at bacterial surfaces is related to invasive infection. *J. Exp. Med.* 209: 2367–2381.
42. Katerov, V., C. Schalén, and A. A. Totolian. 1994. M-like, immunoglobulin-binding protein of *Streptococcus pyogenes* type M15. *Curr. Microbiol.* 29: 31–36.
43. Retnoningrum, D. S., A. Podbielski, and P. P. Cleary. 1993. Type M12 protein from *Streptococcus pyogenes* is a receptor for IgG3. *J. Immunol.* 150: 2332–2340.
44. Otten, R. A., R. Raeder, D. G. Heath, R. Lottenberg, P. P. Cleary, and M. D. Boyle. 1992. Identification of two type IIa IgG-binding proteins expressed by a single group A *streptococcus*. *J. Immunol.* 148: 3174–3182.
45. Kronvall, G., A. Simmons, E. B. Myhre, and S. Jonsson. 1979. Specific absorption of human serum albumin, immunoglobulin A, and immunoglobulin G with selected strains of group A and G streptococci. *Infect. Immun.* 25: 1–10.
46. Heath, D. G., and P. P. Cleary. 1989. Fc-receptor and M-protein genes of group A streptococci are products of gene duplication. *Proc. Natl. Acad. Sci. USA* 86: 4741–4745.
47. Boyle, M. D., J. Weber-Heynemann, R. Raeder, and A. Podbielski. 1995. Characterization of a gene coding for a type IIo bacterial IgG-binding protein. *Mol. Immunol.* 32: 669–678.
48. Courtney, H. S., and Y. Li. 2013. Non-immune binding of human IgG to M-related proteins confers resistance to phagocytosis of group A streptococci in blood. *PLoS One* 8: e78719.
49. Persson, J., B. Beall, S. Linse, and G. Lindahl. 2006. Extreme sequence divergence but conserved ligand-binding specificity in *Streptococcus pyogenes* M protein. *PLoS Pathog.* 2: e47.
50. Ferretti, J. J., W. M. McShan, D. Ajdic, D. J. Savic, G. Savic, K. Lyon, C. Primeaux, S. Sezate, A. N. Suvorov, S. Kenton, et al. 2001. Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc. Natl. Acad. Sci. USA* 98: 4658–4663.
51. Nasser, W., S. B. Beres, R. J. Olsen, M. A. Dean, K. A. Rice, S. W. Long, K. G. Kristinsson, M. Gottfredsson, J. Vuopio, K. Raisanen, et al. 2014. Evolutionary pathway to increased virulence and epidemic group A *Streptococcus* disease derived from 3,615 genome sequences. *Proc. Natl. Acad. Sci. USA* 111: E1768–E1776.
52. Sumby, P., S. F. Porcella, A. G. Madrigal, K. D. Barbian, K. Virtaneva, S. M. Ricklefs, D. E. Sturdevant, M. R. Graham, J. Vuopio-Varkila, N. P. Hoe, and J. M. Musser. 2005. Evolutionary origin and emergence of a highly successful clone of serotype M1 group a *Streptococcus* involved multiple horizontal gene transfer events. *J. Infect. Dis.* 192: 771–782.
53. Talkington, D. F., B. Schwartz, C. M. Black, J. K. Todd, J. Elliott, R. F. Breiman, and R. R. Facklam. 1993. Association of phenotypic and genotypic characteristics of invasive *Streptococcus pyogenes* isolates with clinical components of streptococcal toxic shock syndrome. *Infect. Immun.* 61: 3369–3374.
54. Carapetis, J., R. Robins-Browne, D. Martin, T. Shelby-James, and G. Hogg. 1995. Increasing severity of invasive group A streptococcal disease in Australia: clinical and molecular epidemiological features and identification of a new virulent M-nontypeable clone. *Clin. Infect. Dis.* 21: 1220–1227.
55. Luca-Harari, B., J. Darenberg, S. Neal, T. Siljander, L. Strakova, A. Tanna, R. Creti, K. Ekelund, M. Koliou, P. T. Tassios, et al; Strep-EURO Study Group. 2009. Clinical and microbiological characteristics of severe *Streptococcus pyogenes* disease in Europe. *J. Clin. Microbiol.* 47: 1155–1165.
56. Shulman, S. T., R. R. Tanz, J. B. Dale, B. Beall, W. Kabat, K. Kabat, E. Cederlund, D. Patel, J. Rippe, Z. Li, and V. Sakota, North American Streptococcal Pharyngitis Surveillance Group. 2009. Seven-year surveillance of North American pediatric group a streptococcal pharyngitis isolates. *Clin. Infect. Dis.* 49: 78–84.
57. Gaworzewska, E., and G. Colman. 1988. Changes in the pattern of infection caused by *Streptococcus pyogenes*. *Epidemiol. Infect.* 100: 257–269.
58. Davies, H. D., A. McGeer, B. Schwartz, K. Green, D. Cann, A. E. Simor, and D. E. Low, Ontario Group A Streptococcal Study Group. 1996. Invasive group A streptococcal infections in Ontario, Canada. *N. Engl. J. Med.* 335: 547–554.
59. Aziz, R. K., and M. Kotb. 2008. Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. *Emerg. Infect. Dis.* 14: 1511–1517.
60. Smith, T. C., D. D. Sledjeski, and M. D. Boyle. 2003. Regulation of protein H expression in M1 serotype isolates of *Streptococcus pyogenes*. *FEMS Microbiol. Lett.* 219: 9–15.
61. Nilson, B. H., I. M. Frick, P. Akesson, S. Forsén, L. Björck, B. Akerström, and M. Wikström. 1995. Structure and stability of protein H and the M1 protein from *Streptococcus pyogenes*. Implications for other surface proteins of gram-positive bacteria. *Biochemistry* 34: 13688–13698.
62. Poulsen, H. L. 1974. Interstitial fluid concentrations of albumin and immunoglobulin G in normal men. *Scand. J. Clin. Lab. Invest.* 34: 119–122.
63. Stoop, J. W., B. J. Zegers, P. C. Sander, and R. E. Ballieux. 1969. Serum immunoglobulin levels in healthy children and adults. *Clin. Exp. Immunol.* 4: 101–112.
64. von Pawel-Rammingen, U. 2012. Streptococcal IdeS and its impact on immune response and inflammation. *J. Innate Immun.* 4: 132–140.
65. Collin, M., and A. Olsén. 2001. Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect. Immun.* 69: 7187–7189.
66. Boyle, M. D. P. 1990. *Bacterial immunoglobulin binding proteins.* Academic Press, San Diego, CA.
67. Berge, A., and L. Björck. 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.* 270: 9862–9867.
68. Shannon, O., E. Hertzén, A. Norrby-Teglund, M. Mörgelin, U. Sjöbring, and L. Björck. 2007. Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. *Mol. Microbiol.* 65: 1147–1157.
69. Stevens, D. L. 2001. Invasive streptococcal infections. *J. Infect. Chemother.* 7: 69–80.