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Human IgG increases virulence of *Streptococcus pyogenes* through complement evasion[1]

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Abstract

Streptococcus pyogenes is an exclusively human pathogen, which 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 IgG through its Fc region to the bacterial surface in order to evade immune recognition. We identified a novel virulence mechanism, whereby IgG-enhanced binding of C4BP to five of 12 tested *S. pyogenes* strains that expressed diverse M proteins, which 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 human IgG Fc to protein H increased the affinity of protein H for C4BP. Increased C4BP binding accentuated complement down-regulation resulting in diminished bacterial killing. Accordingly, mortality from *S. pyogenes* infection in human C4BP transgenic mice was increased when human 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

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Conflict of Interest

The authors declare no financial conflict of interest.

paradoxical function of human IgG bound through Fc to diverse *S. pyogenes* isolates, which increases their virulence and may counteract the beneficial effects of IgG opsonization.

Introduction

Streptococcus pyogenes is a commonly encountered and clinically important pathogen (1). Every year *S. pyogenes* infects approximately 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 ten most fatal human pathogens with about 500.000 deaths annually (1). In most individuals, *S. pyogenes* affects the skin or oropharynx but, in some instances, (~650,000 cases world-wide 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 GAS (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 enables 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 present exclusively on M1-expressing *S. pyogenes* strains, 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 serum proteins (6, 7, 21, 26), protein H can also form homodimers (27, 28). Competition between C4BP and human IgG for binding to protein H has been suggested (6, 8). In this study, we characterized the interactions between human IgG, C4BP and protein H and report a novel virulence mechanism of *Streptococcus pyogenes*.

Material and Methods

Bacteria and culture conditions

Streptococcus pyogenes AP1, AP4, AP8, AP15, AP18 (29), AP28, AP29, AP36, AP38, AP43, AP46, AP60, and AP74 (all from the WHO 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), BMJ71 (protein H$^-$ /M$^-$ (32))

were grown in Todd-Hewitt broth (THB) overnight at 37 °C and 5% CO_2. Cultures were then diluted to OD_{600} = 0.1 in fresh THB 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 Table S1.

Proteins and antibodies

For flow cytometric analysis, the following antibodies were used: mouse anti human-C4BP MK104 (33) coupled to biotin; mouse anti-human-FH MRC OX24 (34) coupled to Dylight 647;; goat anti human-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; mouse anti mouse-FH (Hycult) biotin conjugated. Biotin coupled antibodies were stained with streptavidin-PE (eBioscience). Fab and Fc fragments of human IgG were purchased from Calbiochem, human IgG (IVIG; Kiovig) from Baxalta. Mice were administered pooled human Fc fragments (Athens Research), IVIG or denosumab (Amgen) diluted in PBS. Rabbit IgG was purified from pre-immune serum using protein A/G columns. Mouse IgG2a and IgG2b were purchased from Immunotools, goat control IgG from R&D Biosystems. Goat, rhesus and cynomolgus IgG were purchased from Nordic Diagnostica. Human C4BP and FH were purified from human plasma, M18 was purified from culture supernatants using fibrinogen Sepharose, 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 human IgG, as determined by a sandwich ELISA for human 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 3 times with wash buffer (50 mM Tris pH 8.0, 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 gamma counter (Perkin Elmer).

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 1h incubation at 37°C (if not stated otherwise) in 5% CO_2, bacteria were washed 3 times in 1x PBS and radioactivity associated with bacteria detected using a Wizard^2 gamma counter (Perkin Elmer).

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 co-incubated in the presence or absence of 1mg/ml IVIG for 1h at 37°C. To detect C4BP binding to bacteria, C4BP and IVIG were conjugated with colloidal gold. Bacteria were either stained with antibody-Au conjugates or mixed with protein-Au conjugates and incubated for 1 h at 37°C. Five ml 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 OSIS Veleta, 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, which 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 SPF conditions in the animal facility at the University of Massachusetts Medical School, Worcester, USA. All experimental groups were sex and age matched (6–8 weeks old male and female animals, BALB/c and hu-C4BP 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 antibody 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 (1mg human IgG-Fc/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 eight days; 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 human (hu-) C4BP transgenic (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

IVIG, human or mouse C4BP or FH and then analyzed using a Cytoflex (Beckman Coulter) or a Cyflow space flow cytometer (Partec).

Neutrophil killing assay

Human neutrophils 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 (MOI) of 0.1 in the presence of hu-C4BP tg mouse serum and hu-IgG. The neutrophils 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 (SRA), consisting of 3615 runs (accession number SRA036051; https://www.ncbi.nlm.nih.gov/sra?term=SRA036051), using the fastq-dump tool from SRA 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 them. Using RAPsearch version 2.23 (40) (http://sourceforge.net/projects/smalt/) the data 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 Äkta explorer system (GE Healthcare) employing a Superose 6 10/30 column using PBS as eluent; 0.6 ml/min flow at ambient temperature. Proteins were incubated together in PBS for 30 minutes at room temperature before analysis or left untreated as single protein 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 1-way or 2-way ANOVA analysis with Bonferroni's post-test or Mantel Cox (log-rank; to analyze survival) tests as indicated. $P<0.05$ 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 patient with necrotizing fasciitis (in 2006) in whom *S. pyogenes* M1 was identified as the sole pathogen by the clinical microbiology department, 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

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, Worcester, MA, USA 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

Human IgG augments protein H-C4BP binding

S. pyogenes M proteins are known to bind human serum proteins, especially complement inhibitors and immunoglobulins (41–49). In earlier publications, we showed that surface-bound C4BP enhanced virulence of *S. pyogenes* (8, 29). Here we sought, initially, to determine the importance of C4BP binding to *S. pyogenes* more generally in the presence of other human serum components, particularly human 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 human C4BP transgenic mice (6–8, 21, 26–29, 35, 50). AP1 binds C4BP and IgG via protein H, a member of the M protein family. We examined 3,465 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* was similar in its geospatial distribution (Fig.1c; except Iceland) and across invasive and pharyngeal isolates (Fig. 1d–e) during the past 50 years, represented by older pre-resurgence strains before 1980 (Fig. 1f) (51) and more contemporary post-resurgence strains (Fig.1g) (52, 53), respectively.

The binding site for C4BP on protein H resides in close proximity to site where human IgG binds (Fig.2a), suggesting competition between C4BP and IgG binding to protein H (8). Binding of increasing concentrations of ^{125}I-labelled protein H to immobilized C4BP was measured in the presence or absence of 25 μg/ml human IgG (IVIG) at 37°C. IVIG enhanced binding of protein H to C4BP at all concentrations of protein H tested. In the absence of IVIG, 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 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-/multi-merization of protein H.

We next examined the effects of increasing concentrations of IVIG 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-human IgG interaction selectively enhances C4BP binding to protein H

We asked if the protein H-human IgG interaction affected 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, no increase in bound concentration to any of the protein H ligands was measured in the presence of increasing concentrations of IVIG (Fig.2e). Thus, human IgG specifically, increased the binding of protein H only to C4BP (Fig.2d+e). 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 1–2 orders of magnitude greater than binding to other protein H ligands.

We also examined the influence of IVIG 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 due to IgG, while no binding to FH, albumin or α1AT was observed at all (Fig. 2f). Taken together, IgG interacts with protein H to specifically increase the amount of C4BP that 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 human Fc-containing fragments. Non-human IgG did not increase the affinity of protein H for human C4BP (or to a low extent in case of rabbit IgG; Fig.2h). Similar to human IgG, non-human primate IgG increased the amount of C4BP that bound to AP1 (Fig. 2i).

Human IgG forms complexes with protein H and C4BP

To verify the observed protein-protein interaction, we performed size exclusion chromatography (SEC) 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 all 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 protein H, did not bind C4BP independent on 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 less than 10% at 39°C, compared to binding of C4BP to bacteria in the presence of IVIG 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 PEG-coated gold particles of the same size (Fig.S1a–c). We hypothesize that protein H di/multimerizes in the presence of human IgG, which permits greater binding to C4BP (Fig.4f).

Human IgG increases the amount 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 killing of *S. pyogenes* by PMNs. Addition of IVIG to AP1 significantly increased survival at 60 and 90 minutes (Fig.5b), but did not alter survival of AP18 (Fig.5c).

Taken together, these data show that human 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 human IgG increases lethality in mice

AP1 does not bind mouse C4BP or FH (Fig.S2a–b). In contrast, in hu-C4BPxFH transgenic (Tg) mouse serum, binding of hu-C4BP, was augmented ~2-fold upon adding 1 mg/ml of IVIG to the mouse serum (Fig.S2c), while binding of human-FH in Tg mouse serum was not increased upon IVIG addition (Fig.S2d). Interestingly, analysis of binding curves (Fig.S2c)

revealed that adding IgG does not increase the affinity (K_D) of C4BP for AP1 compared to analysis when IgG is absent. However, maximal binding (B_{max}) of C4BP to AP1 nearly doubled when IgG was present, indicating an increased number of binding sites on the bacteria when IgG was bound.

To exclude the effect of Fab directed opsonization *in vivo*, we used IgG-Fc fragments. Hu-C4BP tg mice were treated one day 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 (Fig.S3a). Fc increased C4BP binding to AP1, similarly to whole human IgG (Fig.6b).

To determine if intact human IgG has similar effects on C4BP binding to *S. pyogenes* AP1, we used an unrelated human IgG2 monoclonal antibody, denosumab. Neither denosumab nor goat IgG, opsonized AP1 (Fig.S3c). We pre-treated hu-C4BP tg animals with 2 mg denosumab per animal one day prior to infection (in Day 0), to achieve serum human 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 (Fig.S3b). 90% of the animals treated with denosumab succumbed to infection while all mock treated animals survived (Fig.6c). Similar results were achieved using a different AP1 inoculum (Fig.S3d). Denosumab increased C4BP binding to AP1 *in vitro* (Fig.6d), similar to human IVIG, supporting the role of human 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, on which C4BP binding is not affected by human IgG (Fig.5a). Administration of denosumab did not change mortality in AP18 infected mice compared to mock-treated animals (Fig.6e). Similar to denosumab, AP18 infected hu-C4BP animals treated with IVIG showed no significant difference in survival compared with mock-treated animals (Fig.6f). In contrast, IVIG 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).

IVIG treatment of BALB/c WT mice did not affect survival compared to mock treatment during infection with AP1 (Fig.6h, S3e). Similarly, denosumab did not increase mortality of AP1 infected animals (Fig.S3f), because *S. pyogenes* cannot recruit and utilize mouse C4BP. Goat IgG does not influence C4BP binding to bacteria (Fig.1i) and thus does not alter the course of infection in mice compared to PBS treatment (Fig.S3g).

Taken together, these data show a detrimental effect of human IgG on *S. pyogenes* infection in Tg human 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 patient with necrotizing fasciitis caused solely by *S. pyogenes* expressing 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; Fig.S1d–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 vs. 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–g), whereas it localized with protein H 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 human 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. Thereafter, bound C4BP limits complement activation and reduces opsonization and bacterial elimination by phagocytes. IgG not only increase 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 human IgG enhances *S. pyogenes* infection in mice. Consistent with our findings, we also co-localized 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 availability of C4BP is diminished. Further, 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, 54) including (1, 14, 24, 55–60). Protein H is expressed in 28% (18 of 64) of M1 serotype that cause necrotizing fasciitis, according to an analysis (61) of isolates from a CDC surveillance study (54). We confirmed that 30% (1055/3465) of *S. pyogenes* M1 strains had the *sph* gene encoding protein or a homologue (52).

Because low temperatures induce dimerization of protein H (27, 62) and IgG exhibited a similar effect on protein H-C4BP binding, we speculated 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 human 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 human 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 to serum (63). The concentration of C4BP bound to *S. pyogenes* was maximal at 1 mg/ml IgG (about $1/5^{th}$ −$1/10^{th}$ of serum concentration in healthy adults (64)), consistent with our assertion that *S. pyogenes* has adapted to bind C4BP in environments with low levels of both C4BP and human 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 antibodies binding to their antigenic targets on the streptococcal surface via Fab (41). *S. pyogenes* counteracts Ig-mediated opsonization by secreting enzymes that cleave and inactivate surface-bound IgG (2, 65, 66). It is believed that the binding of IgG antibodies via ‘effector’ Fc fragments to microbes renders them immunologically effete (67), e.g. by preventing Fc-receptors from recognizing IgG-opsonized bacteria (48). Here 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 N-terminus of domain B (6, 8), and could explain why we were unable to identify any strains among 12 tested which 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 human IgG may facilitate bacterial invasion. The virulence mechanism described here 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 (68). Recruitment of C4BP to the released protein H-IgG complex could further enlarge these immune complexes rapidly and contribute to microthromus formation, glomerulonephritis and acute renal failure, which are complications of invasive streptococcal infections (69, 70).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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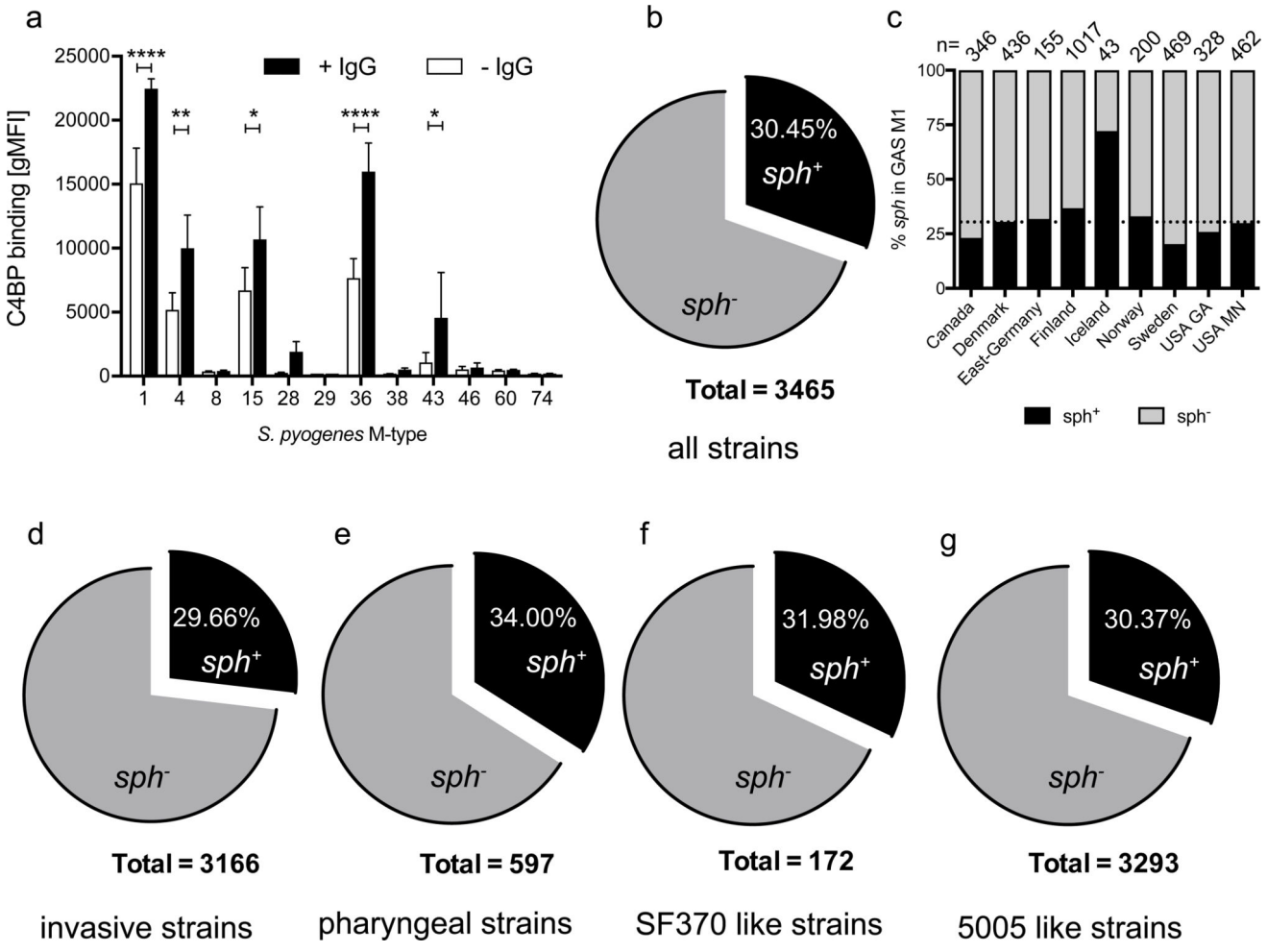

Fig 1. C4BP binding in *S. pyogenes strains and* presence of *sph* in M1

12 different *S. pyogenes* clinical isolates were tested for their C4BP binding in the presence and absence of human 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 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 of 2866 isolates) and in pharyngeal isolates (**e**; 203 of 597 isolates). Interestingly, the distribution of 30% of *sph*+ strains was similar (31.98 % and 30.37%, respectively) among SF370 (**f**; 55 of 172 isolates) and 5005 like M1 strains (**g**; 1000 of 3293 isolates), representing older pre-resurgence 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 years. 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

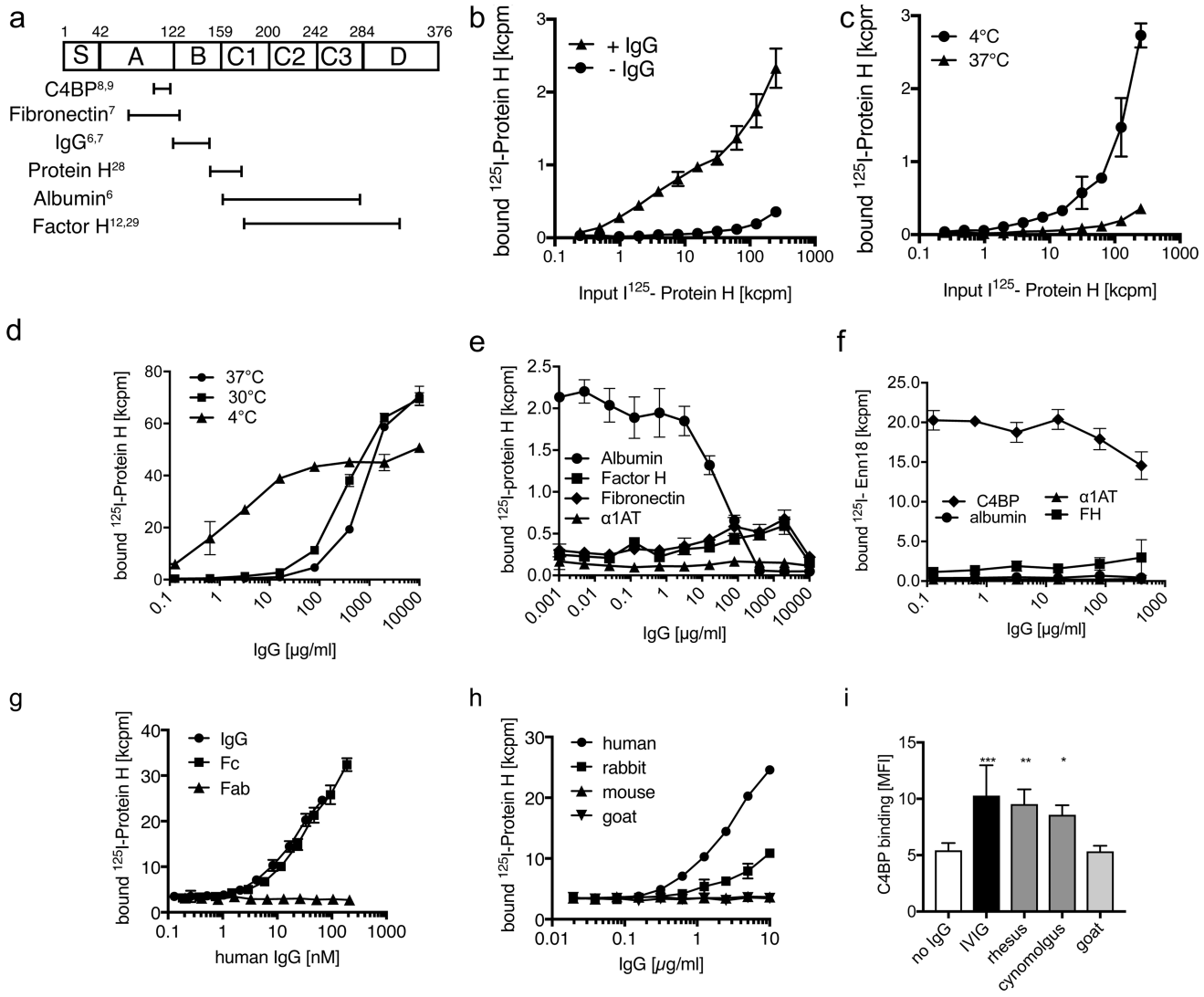

Fig 2. Binding of *S. pyogenes* protein H to human C4BP: effect of human IgG and temperature

(**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 was added to immobilized C4BP at 4°C or 37°C. (**d**) IVIG increases ^{125}I-protein H binding to immobilized C4BP at 4°C, 30°C and 37°C. ^{125}I-protein H (250 kcpm[1]) was incubated with increasing amounts of IVIG. (**e**) ^{125}I- protein H (5–270kcpm) was incubated with increasing amounts of 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 IgG and tested for binding to different serum proteins in the presence of different amounts of IgG. (**g**) Immobilized C4BP (1 µg/ml) was incubated with ^{125}I-protein H (125 kcpm) in the presence of increasing amounts of whole IgG, Fc or Fab fragments. (**h**) Immobilized C4BP was incubated with

[1]kcpm= kilo cpm = 1000 cpm

^{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 were incubated with 1mg/ml IgG from the indicated species in 10% mouse serum containing human C4BP. Mean (±SD) from 3 independent determinations are shown in all experiments. . * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ assessed by 1-way ANOVA. Curve comparison for differences was performed using a 2-way ANOVA: (**b, f, h;** $p<0.0001$); (**d, e**; $p=0.0001$) and (**c;** $p= 0.002$). MFI= mean fluorescence intensity

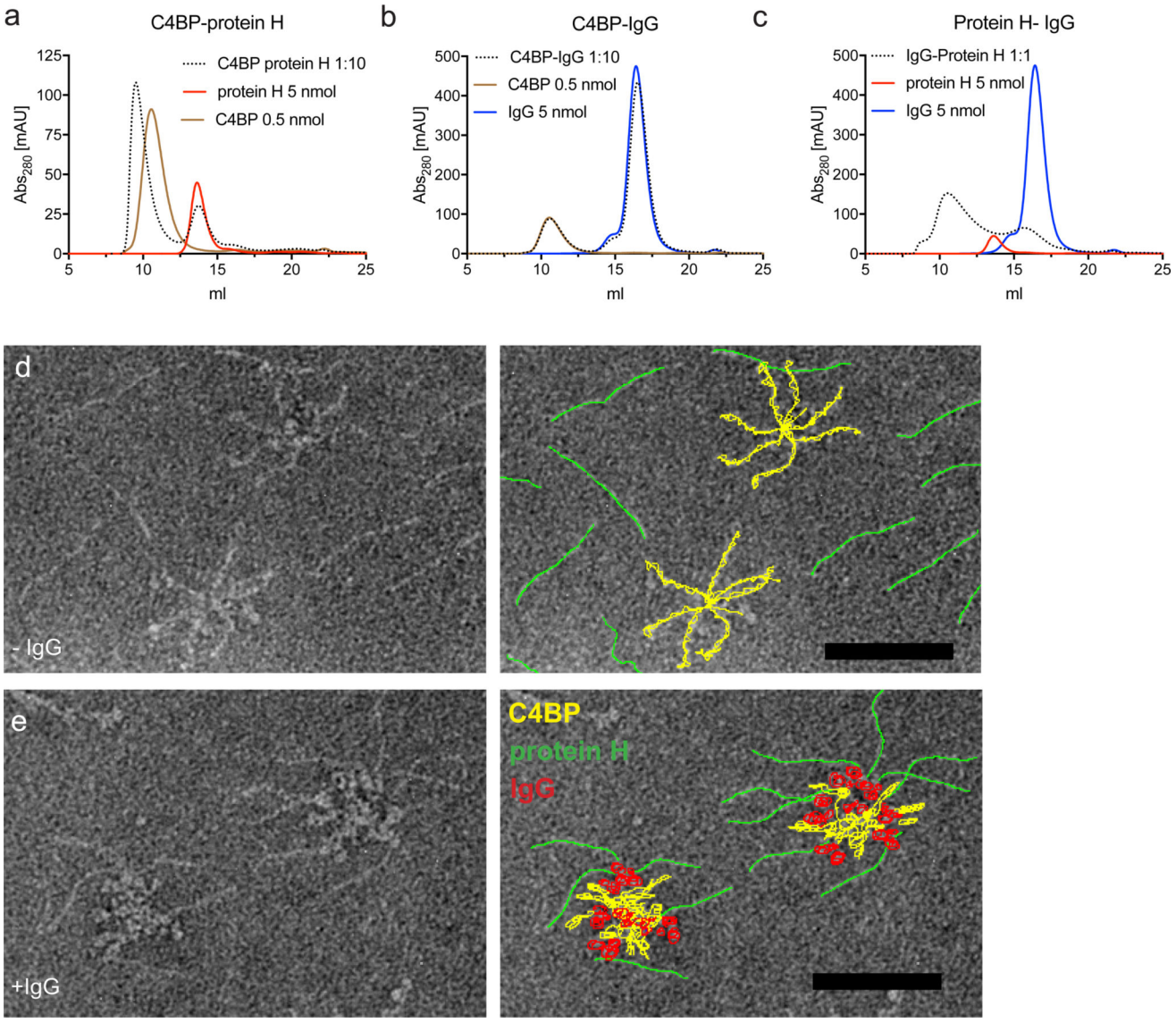

Fig 3. Complex formation of C4BP, protein H and IgG

(**a**) C4BP-protein H (**b**) C4BP-IgG and (**c**) protein H -IgG as well as individual proteins were analyzed by SEC on a Superose 6 column. (**d–e**) Electron microscopy images of negative stained protein complexes formed between IVIG, protein H and C4BP. Proteins were artificially colored: C4BP, yellow; protein H, green and IgG red. Bars (**d-e**) indicate 50 nm. Representative experiments of at least 3 consistent repetitions are shown.

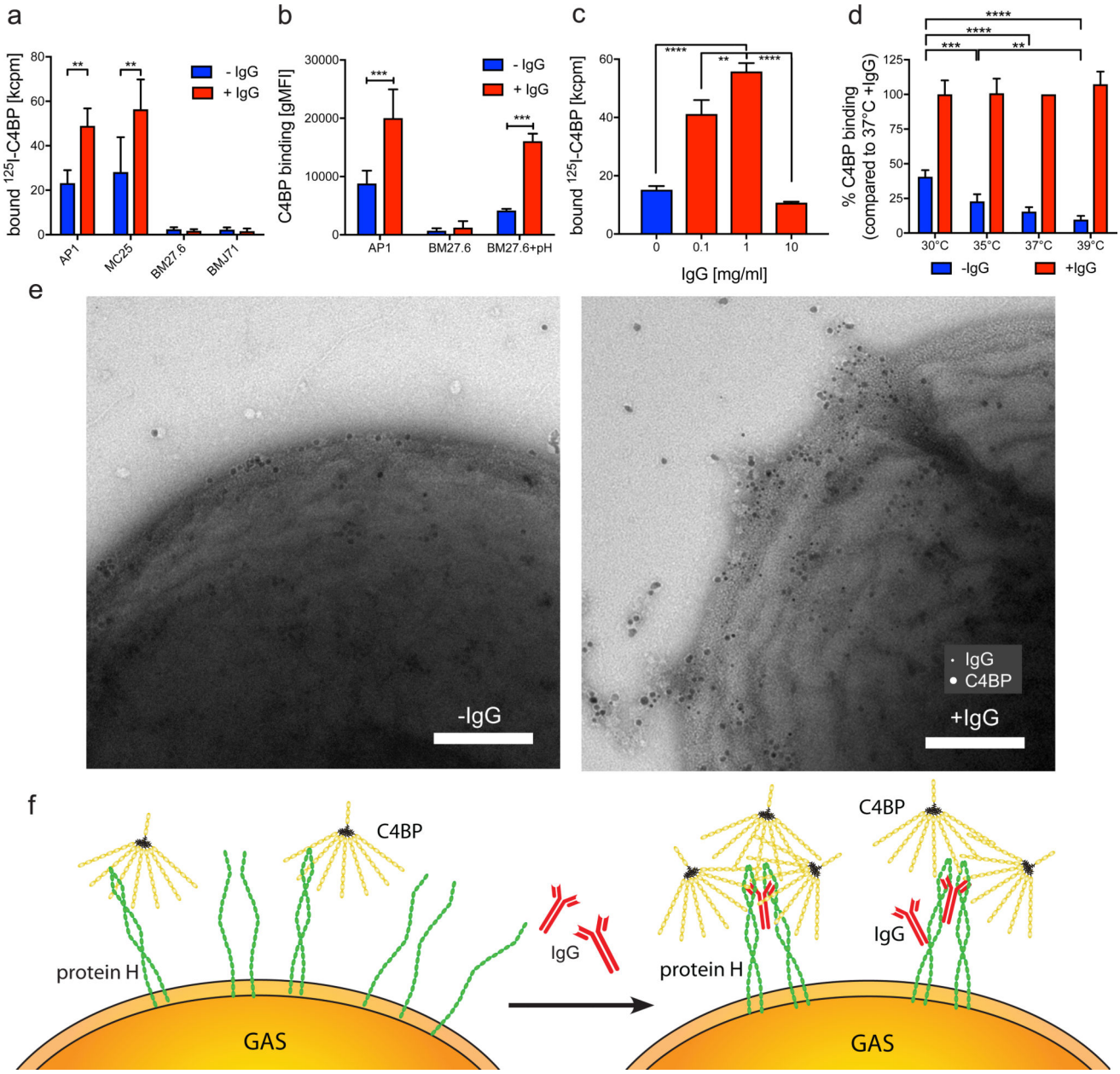

Fig 4. Human IgG increases C4BP-binding to bacteria

(**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 IVIG. (**b**) AP1, BM27.6 and protein H complemented mutant BM27.6+pH were incubated in 10% mouse serum containing human C4BP in the presence or absence of 1mg/ml IVIG. (**c**) AP1 was incubated with ^{125}I-C4BP (100 kcpm) and increasing amounts of IVIG at 37°C. (**d**) AP1 was incubated with in 10% mouse serum containing human C4BP in the presence or absence of 1mg/ml IVIG at the indicated temperatures. (**e**) Electron microscopic confirmation of increased C4BP binding to AP1 in the presence of IVIG. (**f**) Hypothetical model of IgG induced binding of C4BP to protein H. Mean (±SD) from 3 independent determinations are shown. More than 500 interactions from

different areas of the microscopy grids were analyzed. Bar indicates 100nm. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ assessed by 1-way (**c**) or 2-way (**a, b, d**) ANOVA.

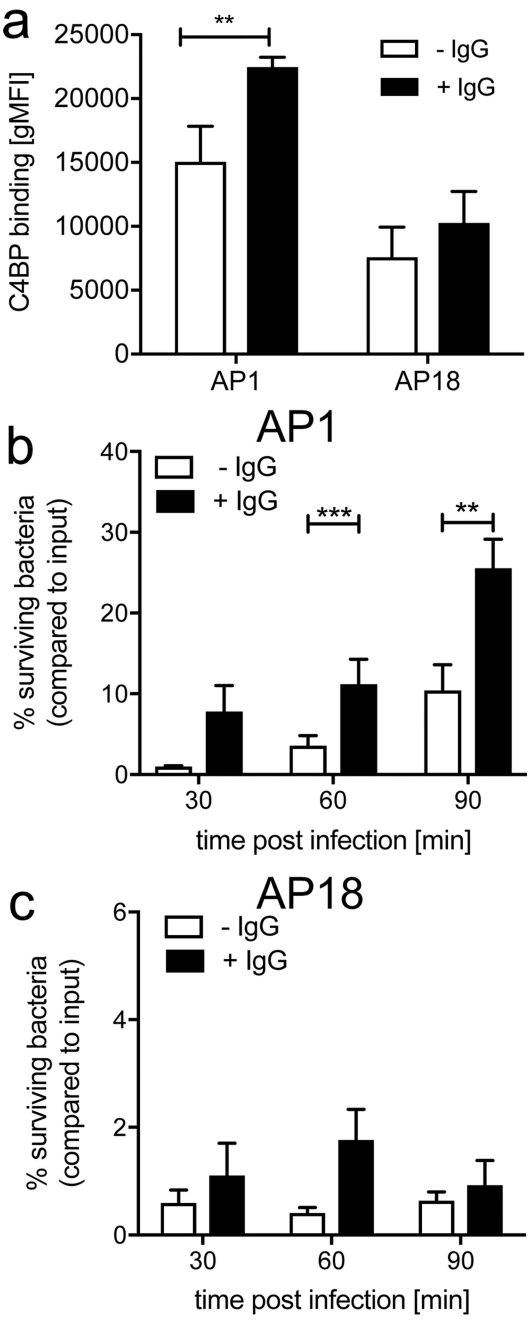

Fig 5. Human IgG enhances C4BP binding to bacteria and promotes bacterial survival

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 IgG. Survival assay of *S. pyogenes* AP1 (**b**) and AP18 (**c**), co-incubated with isolated PMNS and hu-C4BP tg mouse serum in the presence and absence of IgG. Mean (±SD) from 3 independent determinations are shown. Statistical significance was calculated using a 2-way ANOVA with Bonferroni's multiple comparison and **=p<0.01, ***=p<0.001 and ****=p<0.000. Absence of stars indicates no significance. gMFI= geometric mean fluorescence intensity

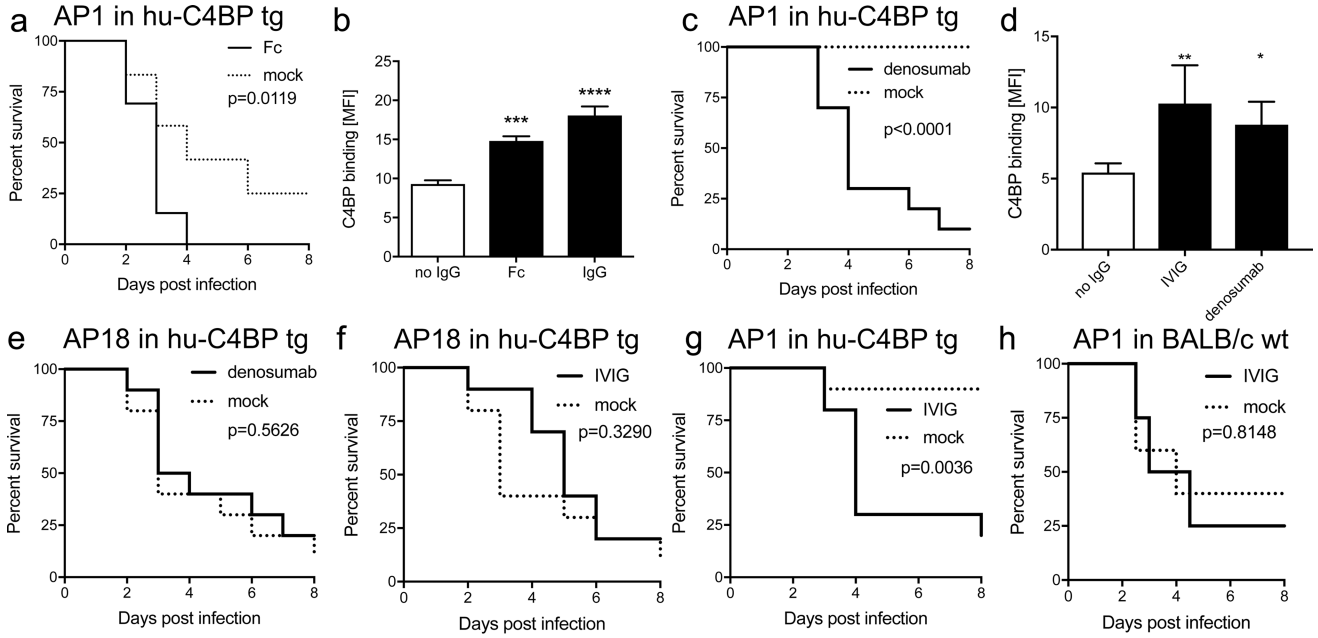

Fig 6. Human IgG increases lethality of *S. pyogenes* infection in hu-C4BP tg mice

(**a**) Hu-C4BP tg animals were injected i.p. with either 1 mg human 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**) Human IgG-Fc fragments exhibit similar effects as intact human IgG (IVIG) on C4BP binding to *S. pyogenes*. (**c, 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 days. (**c**) Hu-C4BP animals (n=10/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–f**) Hu-C4BP tg mice (n=10/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/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 3 independent experiments are shown (**b, d**). Control groups in (**a,h**) received PBS, controls in (**c,e–g**) received goat IgG. Statistical significance was calculated using Mantel-Cox analysis (**a, c, e–h**) and 1-way ANOVA (**b+d**); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ and ****, $p<0.0001$. MFI= mean fluorescence intensity

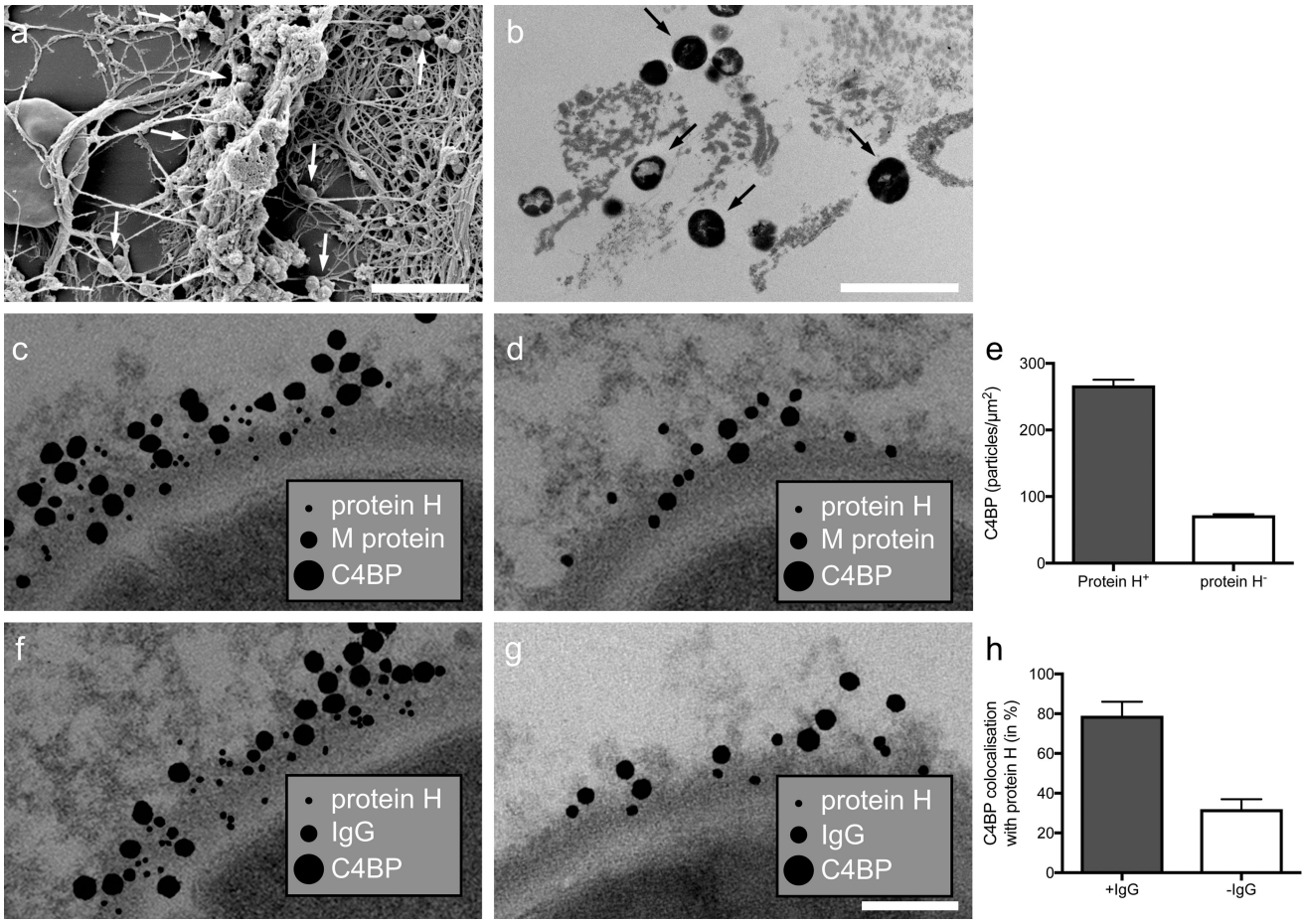

Fig 7. Colocalization of protein H, IgG and C4BP in tissue samples from a patient with necrotizing fasciitis

(**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 antibodies. (**c–d, f–g**) Bacteria are stained either with antibodies coupled to gold particles (size in brackets) that react with C4BP (15nm), (**c–d**) M protein (10nm) or (**f–g**) IgG (10nm) and protein H (5nm). (**c**) *S. pyogenes* M1 that express protein H showed more C4BP (273±38 gold particles/µm^2) compared to 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 to the absence of IgG (32±5%). Bars indicate (**a**) 5 µm, (**b**) 2 µm or (**c–f**) 100 nm. Mean (±SD) of more than 50 bacteria are shown (**e, h**).