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Biofilm Formation Avoids Complement Immunity and Phagocytosis of *Streptococcus pneumoniae*

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Streptococcus pneumoniae **is a frequent member of the microbiota of the human nasopharynx. Colonization of the nasopharyngeal tract is a first and necessary step in the infectious process and often involves the formation of sessile microbial communities by this human pathogen. The ability to grow and persist as biofilms is an advantage for many microorganisms because biofilm-grown bacteria show a reduced susceptibility to antimicrobial agents and hinder the recognition by the immune system. Host protection against biofilm-related pneumococcal disease has not been defined yet. Using pneumococcal strains growing as planktonic cultures or as biofilms, we have investigated the recognition of *S. pneumoniae* by the complement system and its interactions with human neutrophils. Deposition of C3b, the key complement component, was impaired on *S. pneumoniae* biofilms. In addition, binding of C-reactive protein and the complement component C1q to the pneumococcal surface was reduced in biofilm-growing bacteria demonstrating that pneumococcal biofilms avoid the activation of the classical complement pathway. Besides, recruitment of factor H, the down-regulator of the alternative pathway was enhanced by *S. pneumoniae* growing as biofilms. Our results also show that biofilm formation diverts the alternative complement pathway activation by a PspC-mediated mechanism. Furthermore, phagocytosis of pneumococcal biofilms was also impaired. The present study confirms that biofilm formation in *S. pneumoniae* is an efficient way for host immune evasion both from the classical and the PspC-dependent alternative complement pathways.**

Streptococcus pneumoniae, the pneumococcus, is the leading pathogen producing acute otitis media, community-acquired pneumonia, and invasive diseases including bacterial meningitis and sepsis (1). The growth and dispersal of microbes, whether pathogenic or environmental, commonly involve the production of biofilms, which represent the primary mode of pneumococcal growth during colonization, recurrent otitis media and the early stages of invasive disease (2-4). These evidences support the importance of studying pneumococcal sessile communities to understand key events in the pathogenesis development of this important human pathogen.

Biofilm formation is a complex process initiated by the attachment of microorganisms to a surface or interface that is embedded in an extracellular matrix constituted by various polymeric substances (5, 6). The biological and physicochemical characteristics of biofilm structure protect the bacterium from environmental adversities and confer the microorganism an inherent resistance to antimicrobial therapies and the host immune response (6, 7). It is well known that the complement system represents one of the first lines of defense against invading pathogens such as *S. pneumoniae* and plays a vital role in both innate and acquired immunity (8). This unique host defense mechanism is activated by three different pathways —known as the classical, alternative and lectin pathways— that converge at the central component C3, which is involved in essential phases of the immune response such as recognition and clearance of microorganisms, inflammatory response and induction of phagocytosis (8, 9). The classical complement pathway is activated by the recognition of antigen-antibody complexes on the bacterial surface by the complement component C1q and it is generally considered to be an effector of the acquired immune response. This cascade plays a vital role for complement activation against pneumococci (10, 11). However, the classical pathway has also an important role as part of the innate immune response to *S. pneumoniae* since it is activated by other innate immune mediators such as the natural IgM, the C-reactive protein (CRP), the serum amyloid P protein (SAP), or the lectin receptor SIGN-R1 (10-12). Besides, the alternative pathway is activated by the spontaneous hydrolysis of the C3 component, triggering the amplification of C3

deposition and, therefore, contributes significantly to innate immunity (13). In addition, an MBL-independent lectin pathway activation has been recently demonstrated, confirming the importance of complement-mediated immunity against *S. pneumoniae* (14).

Although avoidance of complement immunity and phagocytosis is a clear advantage for bacterial dissemination it may also be a common immune evasion strategy used by selected pathogens to allow long-term colonization and persistent carriage. In this sense, there are several studies reporting the importance of biofilm formation by various microorganisms in the evasion of the host immune response (15-17), although the interactions of pneumococcal biofilms with complement immunity and phagocytic cells is largely unknown.

In this study, we have investigated the recognition by the complement system and human neutrophils of *S. pneumoniae* growing either as biofilms or planktonic cultures by exploring how acute phase proteins and complement down-regulators interact with these two different bacterial life styles.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* non-encapsulated strains used for this study were: strain R6 (a D39 derivative) (18), strain P040 expressing the green fluorescent protein (GFP) [R6 (pMV158GFP) obtained by transformation with plasmid DNA; tetracycline resistant], strain P064 [R6 but *pspC*::*aad9* constructed by mariner mutagenesis, displaying anti-transcribed orientation of the antibiotic resistance cassette of the minitransposon with respect to the targeted gene; spectinomycin-resistant] (19). An encapsulated pneumococcal clinical isolate of serotype 19A (strain 1041) from a patient with sepsis was used. Bacterial strains were grown at 37°C in C medium (20) containing 33 mM potassium phosphate buffer at pH 8.0 (CpH8 medium) either supplemented (or not) with 0.8 mg ml^{-1} yeast extract (C+Y medium). Biofilm formation by pneumococcal cells was obtained using 96-well polystyrene microtiter plates (Costar 3595; Corning) as previously described (19, 21). Briefly, cells were grown in C+Y

medium to an optical density of 0.5–0.6 at 550 nm (OD_{550}), sedimented by centrifugation, resuspended in an equal volume of CpH8 medium, diluted 1/100, and then 200 µl containing 5×10^6 CFU ml^{-1} were dispensed in 96 well plates for biofilm formation or in sterile Falcon tubes for planktonic replication growth. Both cultures were incubated during 5 h at 34°C.

Complement factors binding to *S. pneumoniae* strains. Human serum from five healthy male volunteers unvaccinated against *S. pneumoniae* (median age of 40 years) were obtained with informed consent according to institutional guidelines and stored as single-use aliquots at −70°C as a source of complement and serum components. C1q, C3b, factor H (FH), C4b-binding protein (C4BP) and CRP were assessed using flow cytometry assays as previously described (11). After the incubation process at 34°C, bacterial cultures growing as biofilms in microtiter plates or in the planktonic form were washed with fresh CpH8 medium and resuspended in phosphate-buffered saline (pH 7.0) (PBS). Biofilm disaggregation was performed by gently pipetting and slow vortexing before the opsonization process to avoid possible bias by morphological differences between the two growing stages. The corresponding bacterial suspensions (20 µl) were added to tubes containing 20 µl of human serum diluted 1/5 in PBS and samples were incubated during 20 min at 37°C to allow opsonization by the different serum components. Previously, the number of biofilm-forming CFU was determined by viable counts of bacteria and a similar number of planktonic cells were used in each assay.

C1q and C3b deposition were detected by incubating the bacteria with 50 µl of a fluorescein isothiocyanate- (FITC-) conjugated polyclonal sheep anti-human C1q antibody (Serotec) or a FITC-conjugated polyclonal goat anti-human C3b antibody (ICN-Cappel) diluted 1/300 or 1/500 in PBS/0.1% Tween 20 respectively. Bacterial suspensions and antibodies were incubated during 2 h at 37°C for C1q detection or during 30 min on ice for C3b analysis. The deposition of CRP, FH and C4BP was investigated by using a polyclonal rabbit anti-human CRP antibody (Calbiochem), a polyclonal sheep anti-human FH antibody (Serotec) and a polyclonal sheep anti-human

C4BP antibody (Serotec) for 1 h at 37°C respectively followed by a secondary staining in PBS/0.1% Tween 20 containing FITC-conjugated polyclonal goat anti-rabbit, or FITC/DYLIGHT 649 donkey anti-sheep antibodies (Serotec). After incubations, the bacteria were washed with PBS/0.1% Tween 20 to remove unbound components, fixed in 3% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD Biosciences) using forward and side scatter parameters to gate on at least 25,000 bacteria. The results were expressed as a relative % fluorescence index (FI) that measures not only the proportion of fluorescent bacteria positive for the host serum component investigated but also the intensity of fluorescence that quantify the immune component bound (11, 22).

C3b analysis by confocal laser scanning microscopy. To determine C3b deposition on *S. pneumoniae*, the R6 strain was first grown as biofilm and planktonic culture as described above using glass-bottom dishes (WillCo-dish; WillCo Wells B. V., The Netherlands) or sterile Falcon tubes, respectively. Pneumococcal biofilm and planktonic culture samples were incubated with human serum as a source of complement and the different samples were incubated during 20 min at 37°C to allow opsonization by the C3b component. A FITC-conjugated polyclonal goat anti-human C3b antibody (ICN-Cappel) was used to detect the C3b bound using a Leica TCS-SP5-AOBS-UV confocal laser scanning microscope (CLSM) equipped with an argon ion laser and a Leica DM4000B fluorescence microscopy. Pneumococcal cells were labeled with SYTO 59. The planktonic cells were sedimented by centrifugation to improve the visualization the C3b component by fluorescence microscopy. The excitation/emission maxima were around 495/519 and 622/645 nm for anti-human C3b-FITC and SYTO 59, respectively, and the magnification was ×100. Images were analyzed using the Leica software LCS. Projections through the *x-y* plane (individual scans at 0.5-µm intervals) and the *x-z* plane (images at 3-µm intervals) were obtained by CLSM.

Quantification of IgG, phosphorylcholine (PCho) and PspC: Detection of IgG, Pcho or PspC on the bacterial surface of biofilms and planktonic cultures was determined by flow cytometry. Briefly, the experimental conditions of the assay were

the same than explained above except that bacterial strains were incubated during 20 minutes at 37°C with 50% human serum as a source of IgG, or during 1 h at 37°C with antibodies TEPC-15 (monoclonal antibody to PCho, Sigma-Aldrich) diluted 1/25 or rabbit polyclonal antibody to PspC diluted 1/300 (a kind gift from Prof Sven Hammerschmidt, University of Greifswald, Germany). The secondary antibodies used were rabbit anti-mouse FITC (Serotec) or goat anti-rabbit FITC (Serotec) for the detection of PCho or PspC respectively. Detection of IgG by flow cytometry was measured after incubation for 20 minutes on ice with a Phycoerithryn- (PE) -conjugated donkey anti-human IgG antibody (Jackson Immunoresearch). All secondary antibodies were diluted 1/200 in PBS-Tween 20 (0.1%) and incubated for 30 minutes on ice.

Phagocytosis of *S. pneumoniae* biofilms and planktonic cultures. Experiments investigating human neutrophil phagocytosis were performed by a flow cytometry assay using HL-60 cells (CCL-240; ATCC) differentiated to granulocytes (11, 23). The assay included the fluorescent pneumococcal P040 strain grown as biofilm or planktonic culture in CpH8 medium containing 1% maltose and 1 μg ml^{-1} tetracycline (19). Briefly, 96-well plates containing 1×10^6 CFU per well were infected in triplicate at a ratio of 10 bacteria:1 cell with 20 μl of a suspension of the pneumococcal P040 strain previously opsonized for 20 minutes with HBSS, heat inactivated serum (HIS) or the human serum and the mixture containing cells was incubated for 30 minutes at 37°C with shaking (150 rpm). A minimum of 6,000 cells were analyzed using a Cytomics FC500 Beckman Coulter flow cytometer equipped with a 488 nm Ar-ion laser. The presence of complement receptors on HL-60 granulocytes has been previously documented and therefore expression of CD11b (iC3b receptor and CR3 α-chain), a marker of granulocytic differentiation, was measured prior to phagocytic assays to confirm the presence of the receptor (24). Results were expressed as a FI (see above) defined as the proportion of positive cells for fluorescent bacteria multiplied by the geometric mean of fluorescence intensity which correlates with the amount of bacteria phagocytosed per cell (11, 23).

Statistical analysis. Data are representative of results obtained from repeated independent experiments, and each data point represents the mean and standard deviations (SD) for 3 to 5 replicates. Statistical analysis was performed by using two-tailed Student´s *t* test (for two groups). GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA) was used for statistical analysis.

RESULTS

C3b deposition on *S. pneumoniae* growing as biofilms or planktonic cultures. The deposition of the complement component C3b on the surface of *S. pneumoniae* was investigated by a flow cytometry assay using bacteria grown either as biofilms or as planktonic cultures. Since non-encapsulated pneumococci show a higher capacity to form *in vitro* biofilms than encapsulated isolates (reviewed in reference (6)) and to prevent any possible hindrance of the capsular polysaccharide on complement activity (12), the non-encapsulated pneumococcal R6 strain was used. In addition, to avoid possible bias in complement interaction with *S. pneumoniae* between the two modes of bacterial growth, biofilm disaggregation was performed before opsonization with human serum. The morphologies of *S. pneumoniae* cells from planktonic cultures or from disaggregated biofilms (mainly, diplococci) were indistinguishable by phase contrast microscopy confirming that disaggregation does not induce morphological changes that could affect complement interaction in further assays (not shown). Recognition of *S. pneumoniae* by the key complement component C3b was explored by flow cytometry using a pneumococcal strain without capsule (Figs. 1A and B) and an encapsulated clinical isolate of serotype 19A (Figs. 1C and D). C3b deposition on pneumococcal biofilms was markedly impaired in comparison to planktonic cultures suggesting that biofilm formation in *S. pneumoniae* is a mechanism used by the bacterium to avoid the recognition by this key complement component (Fig. 1). C3b binding was further investigated on planktonic cultures and intact biofilms of the R6 strain using fluorescence microscopy and CLSM, respectively (Fig. 2). C3b bound on the bacterial surface was detected by using FITC-conjugated polyclonal goat anti-

223 human C3b antibody (green fluorescence), whereas the pneumococcal cells were
224 stained with SYTO 59 (red fluorescence). The entire bacterial surface of the planktonic
225 culture was coated by C3b (Figs. 2A and B), whereas only small patches of the
226 pneumococcal biofilm appear to contain C3b (Figs. 2C and D). This confirmed that
227 when *S. pneumoniae* cells form biofilms a notable reduction of the opsonization process
228 by C3b occurs.

229 **Reduced activation of the classical complement pathway by pneumococcal**
230 **biofilms.** As the classical pathway is essential for complement activation against *S.*
231 *pneumoniae* (see above), deposition of its first component, C1q, on the R6 strain was
232 investigated by flow cytometry. C1q deposition was significantly reduced on the surface
233 of pneumococcal biofilms compared to planktonic cultures (Figs. 3A and B), indicating
234 that pneumococcal biofilms hinder the activation of the classical pathway. Since
235 recognition of *S. pneumoniae* by the pentraxin CRP (an acute phase protein) increases
236 the deposition of C1q on the pneumococcal surface activating therefore the classical
237 pathway (12) we tested whether the reduced C1q level on the surface of pneumococcal
238 biofilms was somewhat mediated by an impaired binding by human CRP on biofilms.
239 Actually, binding to human CRP was strongly reduced on the surface of *S. pneumoniae*
240 R6 biofilms in comparison to planktonic cultures (Figs. 3C and D). These results taken
241 together demonstrate that pneumococcal biofilms enhance the resistance of *S.*
242 *pneumoniae* to complement immunity by diminishing the classical pathway activation.
243 Additional experiments confirmed that the impaired recognition of *S. pneumoniae*
244 biofilms by C1q and CRP was not related to differences in binding to IgG (Figs. 3E and
245 F) or variations in the amount of the PCho epitope on the bacterial surface (Figs. 3G and
246 H).

247 **Recruitment of human complement regulators.** Interaction of pneumococcal
248 cultures grown as biofilms or planktonic cultures with the major fluid-phase regulators
249 of either the classical/lectin (C4BP) or alternative (FH) complement cascades was
250 investigated by flow cytometry (Fig. 4). Deposition of C4BP was very similar in
251 planktonic and biofilm cultures indicating that interaction with the main down-regulator

of the classical pathway is not affected by biofilm formation (Figs. 4A and B). In contrast, recruitment of FH was significantly enhanced on pneumococcal biofilms compared to planktonic cultures (Figs. 4C and D), which suggested that the impairment of the alternative pathway in pneumococcal biofilms is mediated by an increased binding to FH, the down-regulator of the alternative cascade.

PspC is involved in the enhanced resistance of pneumococcal biofilms to complement-mediated immunity. It has been documented that the pneumococcal surface protein PspC (also designated CbpA) is able to bind FH (25-27). To explore the possible involvement of PspC in the increased recruitment of FH on pneumococcal biofilms, an isogenic *pspC* mutant of the R6 strain (P064 strain) was employed. Indeed, loss of PspC expression in P064 cells growing as biofilms caused a decrease on FH binding to levels similar to those shown by planktonic cultures of the same strain (Figs. 4E, F, and G). C3b deposition assays using biofilms of the wild-type and *pspC* deficient strain were performed demonstrating that the increased recruitment of FH mediated by PspC confers an advantage to *S. pneumoniae* growing as biofilms to avoid the recognition by C3b (Figs. 5A and B). However, in the absence of PspC in both biofilms and planktonic cultures, a similar C3b deposition pattern was found confirming that this protein is clearly involved in the enhanced resistance of pneumococcal biofilms to the complement system (Figs. 5C and D). To explore the possibility that *S. pneumoniae* growing as biofilms might display greater levels of PspC to avoid complement mediated immunity, detection of the PspC exposed on the bacterial surface was analyzed in pneumococcal biofilms and planktonic cultures (Figs. 5E and F). Our results showed increased levels of PspC on the surface of the biofilm confirming that *S. pneumoniae* adopting a sessile life divert the amplification of the alternative pathway and consequently the deposition of C3b by inducing higher levels of PspC on the bacterial envelope.

Phagocytosis by neutrophils is impaired in *S. pneumoniae* biofilms. Activation of complement immunity is a very efficient mechanism of the host immune response involved in phagocytosis of pneumococci and other encapsulated bacteria. The

susceptibility of pneumococcal biofilms to the opsonophagocytosis mediated by human neutrophils was investigated by flow cytometry using strain P040. Uptake of *S. pneumoniae* grown as a biofilm was markedly impaired in comparison to the planktonic culture demonstrating that the sessile growth of *S. pneumoniae* represents a benefit to the microorganism by avoiding very efficiently the phagocytosis mediated by human neutrophils (Fig. 6). However, in the absence of complement, (HBSS or heat inactivated serum) phagocytosis levels were drastically reduced in comparison to bacteria opsonized with serum confirming that the increased resistance to phagocytosis by pneumococcal biofilms is complement dependent (Fig. 6). Overall, these findings mirror the results obtained above regarding the interaction with different components of the complement immune response and strongly suggest that the reduced complement activation on the surface of pneumococcal biofilms confers the bacterium an enhanced resistance to be phagocytosed by professional phagocytes.

DISCUSSION

Bacterial biofilms are widely accepted as a frequent cause of chronic persistent infections (5). The ability of respiratory pathogens to persist in the nasopharynx and disseminate throughout the host under certain favorable circumstances, is associated with their biofilm-forming capacity on the mucosal epithelium (7). Nasopharyngeal colonization provides a stable environment to *S. pneumoniae* from which it can spread to other hosts and/or give rise to an infection (28). Compared to their planktonic counterparts, bacteria living as biofilms appear to have developed an evolutionary advantage because they are less sensitive to antibiotics, which complicates the effectiveness of the antimicrobial therapy (7, 29). Two main questions that remain unanswered to date is how the host defense immune system reacts to *S. pneumoniae* biofilms and whether or not pneumococcal biofilms can be efficiently recognized by professional phagocytes. One of the major immunological mechanisms against microbial pathogens is complement-mediated immunity that consists of a complex network of circulating and cell surface-bound proteins that play an essential role in host

309 defense (8, 9). In this sense, it has been shown that biofilm formation by *Mycoplasma*
310 *pulmonis* protects from the lytic effects of complement immunity (16) whereas
311 *Staphylococcus epidermidis* growing as biofilms have developed the possibility of
312 avoiding neutrophil killing by preventing C3b opsonization (30). In this study, we have
313 investigated the interaction of *S. pneumoniae* with the complement system exploring the
314 activation and regulation of complement immunity on biofilm and planktonic bacteria.
315 Our results indicate that pneumococcal cells within biofilms are much more effective in
316 diverting C3b deposition on the bacterial surface than planktonic bacteria. This is
317 relevant from the immunological perspective because C3b is essential for both the
318 innate and adaptive immunity against pyogenic bacteria such as *S. pneumoniae* (8, 10,
319 11).

320 To unravel the mechanism behind the impaired C3b deposition on pneumococcal
321 biofilms, the classical pathway activation was investigated, as this cascade is essential
322 for complement immunity against pneumococcus (10, 11). Pneumococcal biofilms have
323 been identified in children with acute otitis media (2) and therefore, the impaired
324 classical pathway activation on *S. pneumoniae* biofilms may have functional
325 consequences, as C1q protects not only from pneumococcal pneumonia and sepsis (10)
326 but also from acute otitis media and invasive disease by avoiding the dissemination of *S.*
327 *pneumoniae* from the middle ear to the systemic circulation (31). Our results
328 demonstrate that biofilm formation confer to *S. pneumoniae* an enhanced ability to
329 circumvent the early activation of this pathway by a C1q-dependent mechanism, which
330 is consistent with previous results reported with *Acinetobacter baumannii* (17).

331 The classical pathway can also be activated on *S. pneumoniae* by acute phase proteins
332 such as CRP (22). CRP is the main acute phase reactant in humans and indeed, CRP
333 levels markedly increase after pneumococcal infection, which confirms the importance
334 of this molecule for *S. pneumoniae* recognition (32). Our findings show that biofilm
335 formation in *S. pneumoniae* is associated with a reduced recognition by human CRP.
336 This is in agreement with that previously reported for coagulase-negative
337 staphylococcal biofilms (33) but contrasts with the claim that an enhanced production of

choline phosphate, which is known to bind CRP, occurs during biofilm development (34). Our results suggest that the impaired activation of the classical pathway on the surface of pneumococcal biofilms is not due to differences in phosphorylcholine or variation in the binding to IgG. There are evidences confirming that CRP binds the complement component C1q through its globular head region activating therefore the classical pathway (35, 36). In this sense, our results indicate that *S. pneumoniae* growing as biofilms have the ability to avoid the direct interaction of C1q with the pneumococcal surface as a recent study has demonstrated that C1q can directly recognize *S. pneumoniae* in the absence of any mediator (37).

Recruitment of regulators for complement activation is a common strategy used by different microorganisms for complement evasion (38). The PspC protein of *S. pneumoniae* binds FH reducing the activation of the alternative complement pathway (27). Our results demonstrated that recruitment of FH was markedly enhanced by pneumococcal biofilms compared to their planktonic counterparts in a PspC-dependent manner and are in accordance with a recent report showing that pneumococci increase the production of PspC when grown under biofilm forming conditions (34). Our findings demonstrate that the increased presence of PspC on the surface of pneumococcal biofilms have functional consequences in terms of subversion of complement mediated immunity by reducing the activation of the alternative pathway through a FH dependent mechanism. In contrast, a significant difference in the deposition of C4BP onto biofilms as compared to planktonic pneumococcal cultures was not apparent. A variety of bacteria interact with C4BP to facilitate immune evasion (reviewed in reference (39). It has been reported that the binding of C4BP to *S. pneumoniae* is PspC allele-dependent, being the R6/D39 allele a weak binder (40). More recently, however, it has been reported that the pneumococcal glycolytic enzyme enolase, a moonlighting surface protein (41), recruited C4BP, but not FH (39). Previous proteomic analyses using procedures different to that employed in this study to grow pneumococcal biofilms have show either a transient increase (42) or a marked inhibition in enolase biosynthesis (43). This discrepancy has been attributed to the different strains

used, the different age of the biofilms examined, and/or to differences in the criteria used for protein identification in both studies. Assuming that significant changes on the binding of C4BP to biofilm- and planktonic-grown cells have not been found, we propose that no major alterations on enolase production take place under our experimental conditions. Overall, our study shows for the first time that *S. pneumoniae* biofilms avoid complement immunity by targeting both the classical and alternative pathway using a complex mechanism of impaired activation and increased down-regulation, respectively.

Clearance of *S. pneumoniae* by professional phagocytes requires an efficient opsonization of the bacterium by the complement system (8). Biofilm formation has been suggested to be a pivotal event in the pathogenesis process of numerous infectious diseases (5) and is consistent with our findings as long as the reduced complement activation on *S. pneumoniae* biofilms confers a significant benefit to the virulence of the microorganism. Reduced phagocytosis has been previously documented for other bacterial species growing as biofilms, confirming that sessile communities of certain microbial pathogens are more resistant to opsonic killing by host phagocytes than planktonic cells (15, 44, 45). In the case of *S. pneumoniae*, biofilm matrices consist of a mixture of extracellular polymeric substances composed of extracellular DNA, proteins and polysaccharides that are synthesized in large part by the pneumococcal strains producing the biofilm. The relevance of these matrices is because they are responsible for the cohesion and three-dimensional architecture of biofilms (46). In terms of host-pathogen interaction, the presence of an extracellular matrix during biofilm formation improves the virulence of *S. pneumoniae* (47). The results of our study fully confirm that opsonophagocytosis of pneumococcal biofilms was significantly impaired in comparison to the planktonic cultures and demonstrate that pneumococcal biofilms have developed an increased resistance to the phagocytosis process mediated by human neutrophils. Taken together, our findings suggest that biofilm formation may constitute an evolutionary advantage in certain phases of the pneumococcal pathogenic process,

such as nasopharyngeal colonization or during the early steps of microbial attachment for invasion, by avoiding the host immune system.

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559 **FIGURE LEGENDS**

560

561 **FIG 1** C3b deposition on the surface of the R6 strain grown as planktonic culture (PK)
562 or as a biofilm (BF) (A). Results are expressed as a relative % FI relative to the results
563 for PK culture. (B) Example of a flow cytometry histogram for C3b deposition. A
564 control (CT PBS) incubated with PBS instead of human serum is also shown. (C) C3b
565 deposition on the surface of the encapsulated clinical isolate of serotype 19A grown as
566 planktonic culture (PK) or as a biofilm (BF) (D). Results are expressed as a relative %
567 FI relative to the results for PK culture. Error bars represent standard deviations and
568 asterisks mark results that are statistically significant compared to bacteria growing as
569 PK (two-tailed Student's *t* test, **P* <0.001).

570

571 **FIG 2** C3b deposition on the surface of the R6 strain grown as planktonic cultures or as
572 biofilms. A planktonic culture of R6 strain was stained with SYTO 59 (A, red) and C3b
573 deposition on the surface of the planktonic culture of the R6 strain was visualized using
574 a FITC-conjugated polyclonal goat anti-human C3b antibody (B, green). To enhance the
575 quality of the picture, the culture was centrifuged and gently resuspended in PBS after
576 labeling and prior to examination at the fluorescence microscope (C–E) Localization by
577 CLSM of the human C3b component on the surface of biofilm-grown R6 strain. A
578 biofilm of the *S. pneumoniae* strain R6 was stained with a combination of SYTO 59 (C,
579 red) and a FITC-conjugated polyclonal goat anti-human C3b antibody (D, green). Image
580 (E) is a merger of the two channels. Scale bars = 25 μm.

581

582 **FIG 3** Deposition of C1q, CRP and IgG on the surface of the R6 strain grown as
583 planktonic culture (PK) or as biofilm (BF) and detection of PCho in PK and BF
584 cultures. (A, C and E) Deposition of C1q, CRP and IgG respectively. (B, D and F)
585 Examples of flow cytometry histograms for the binding of C1q, CRP and IgG
586 respectively. (G) Detection of PCho on the surface of the R6 strain grown as planktonic
587 culture (PK) or as biofilm (BF). (H) Example of a flow cytometry histogram for the

588 detection of PCho. Results are expressed as a relative % FI relative to the results for PK
589 culture. Controls incubated with PBS instead of human serum (CT PBS) are also shown.
590 Error bars represent standard deviations and asterisks mark results that are statistically
591 significant compared to bacteria growing as PK (two-tailed Student's *t* test, **P* <0.001).
592
593 **FIG 4** Recruitment of down-regulators of the complement system by the R6 strain
594 grown as planktonic culture (PK) or as a biofilm (BF). (A) Recruitment of C4BP by the
595 R6 strain as PK (open bar) or as BF (grey bar). (B) Example of a flow cytometry
596 histogram for the deposition of C4BP on R6 strain. (C) Recruitment of FH by the R6
597 strain as PK (open bar) or as BF (grey bar). (D) Example of a flow cytometry histogram
598 for the deposition of FH on R6 strain. (E-F) Recruitment of FH by the P064 (R6 *pspC*)
599 strain as PK (open bar) or as BF (grey bar). (G) Example of a flow cytometry histogram
600 for the recruitment of FH on P064 strain. Results are expressed as a relative % FI
601 relative to the results for PK culture except in panel F, where results indicate the mean
602 fluorescence intensity. Error bars represent standard deviations and asterisks mark
603 results that are statistically significant compared to bacteria growing as PK (two-tailed
604 Student's *t* test, **P* <0.001). Controls incubated with PBS instead of human serum are
605 also shown (CT PBS).
606
607 **FIG 5** Effect of PspC in complement evasion and levels of PspC in planktonic culture
608 (PK) and biofilm (BF). (A) Deposition of C3b on the surface of the R6 strain or its
609 isogenic *pspC* mutant strain growing both as biofilms**.** (B) Example of a flow cytometry
610 histogram for the deposition of C3b on R6 and P064 (R6 *pspC*) strains as BF. (C)
611 Deposition of C3b on the surface of the *pspC* strain growing as PK or BF. (D) Example
612 of a flow cytometry histogram for the deposition of C3b on P064 (R6 *pspC*). (E)
613 Detection of PspC on the bacterial surface of R6 strain growing as PK or as BF. (F)
614 Example of a flow cytometry histogram for the detection of PspC on R6 growing as PK
615 or as BF. Error bars represent standard deviations and asterisks mark results that are
616 statistically significant compared to bacteria growing as PK (two-tailed Student's *t* test,

617 *P <0.001). Controls incubated with PBS instead of human serum are also shown (CT
618 PBS).
619
620 **FIG 6** Opsonophagocytosis of the R6-GFP strain grown as planktonic culture (PK) or
621 as a biofilm (BF). (A) Opsonophagocytosis of the P040 strain as PK (open bar) or as BF
622 (grey bar). Negative controls of bacteria incubated with HBSS or heat inactivated serum
623 (HIS) instead of human serum are also shown. (B) Example of a flow cytometry
624 histogram for the opsonophagocytosis. Results are expressed as a relative % FI relative
625 to the results for PK culture. Error bars represent standard deviations and asterisks mark
626 results that are statistically significant compared to bacteria growing as PK (two-tailed
627 Student's *t* test, *P <0.001).
628
629

Figure 1

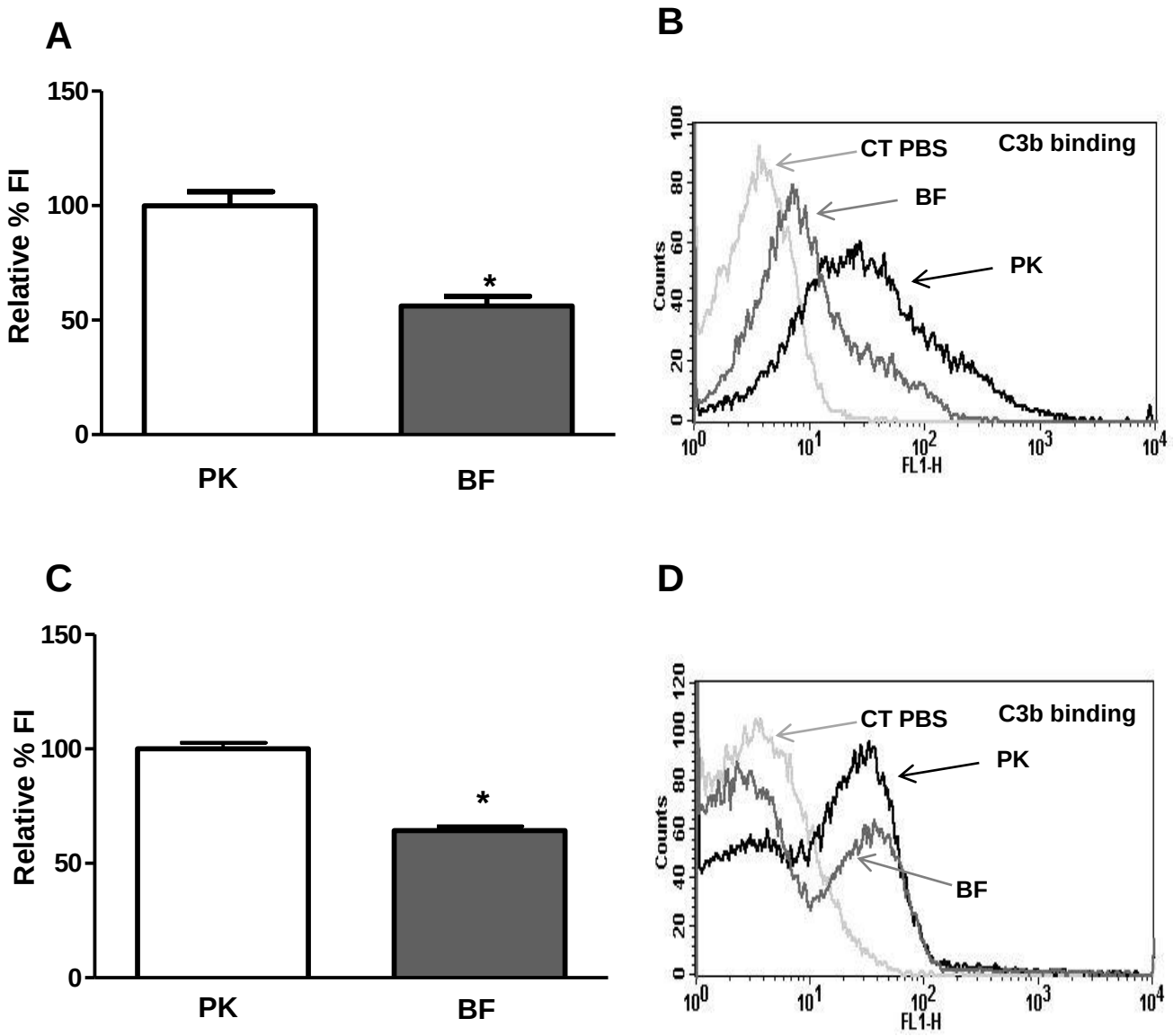
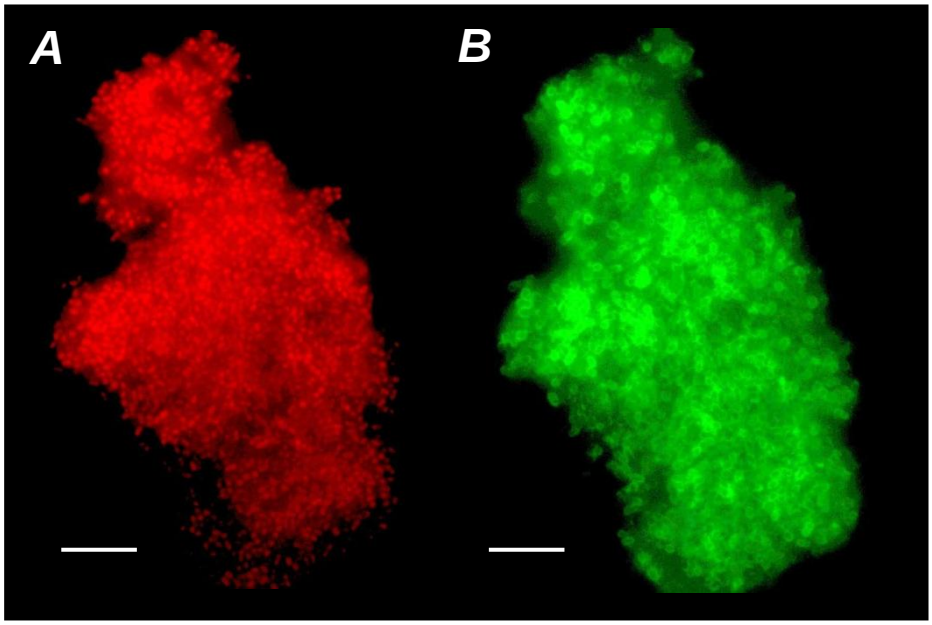
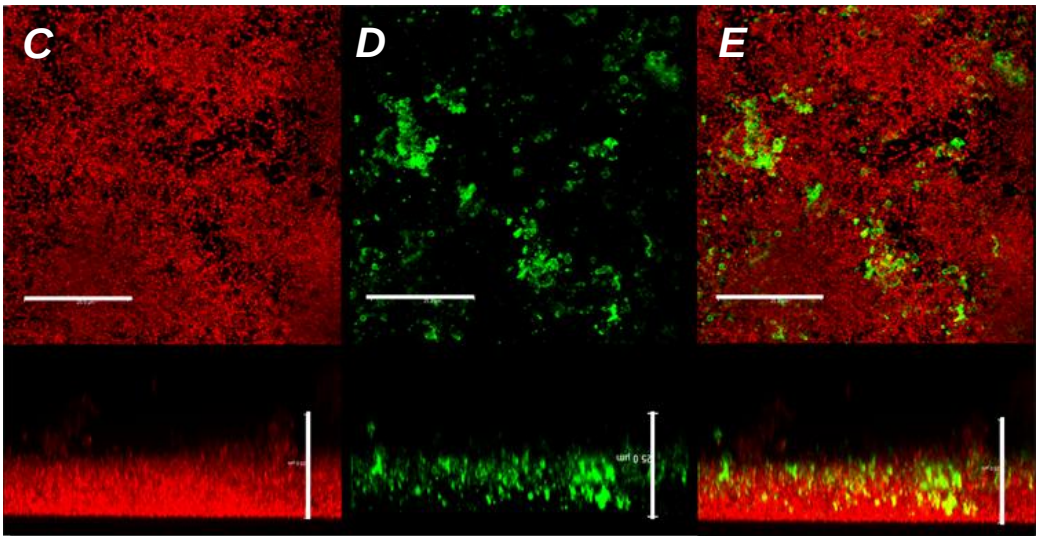

A
150
100
50
0
Relative % FI
*
PK
BF
B
CT PBS
C3b binding
BF
PK
Counts
FL1-H
C
150
100
50
0
Relative % FI
*
PK
BF
D
CT PBS
C3b binding
PK
BF
Counts
FL1-H

Figure 2





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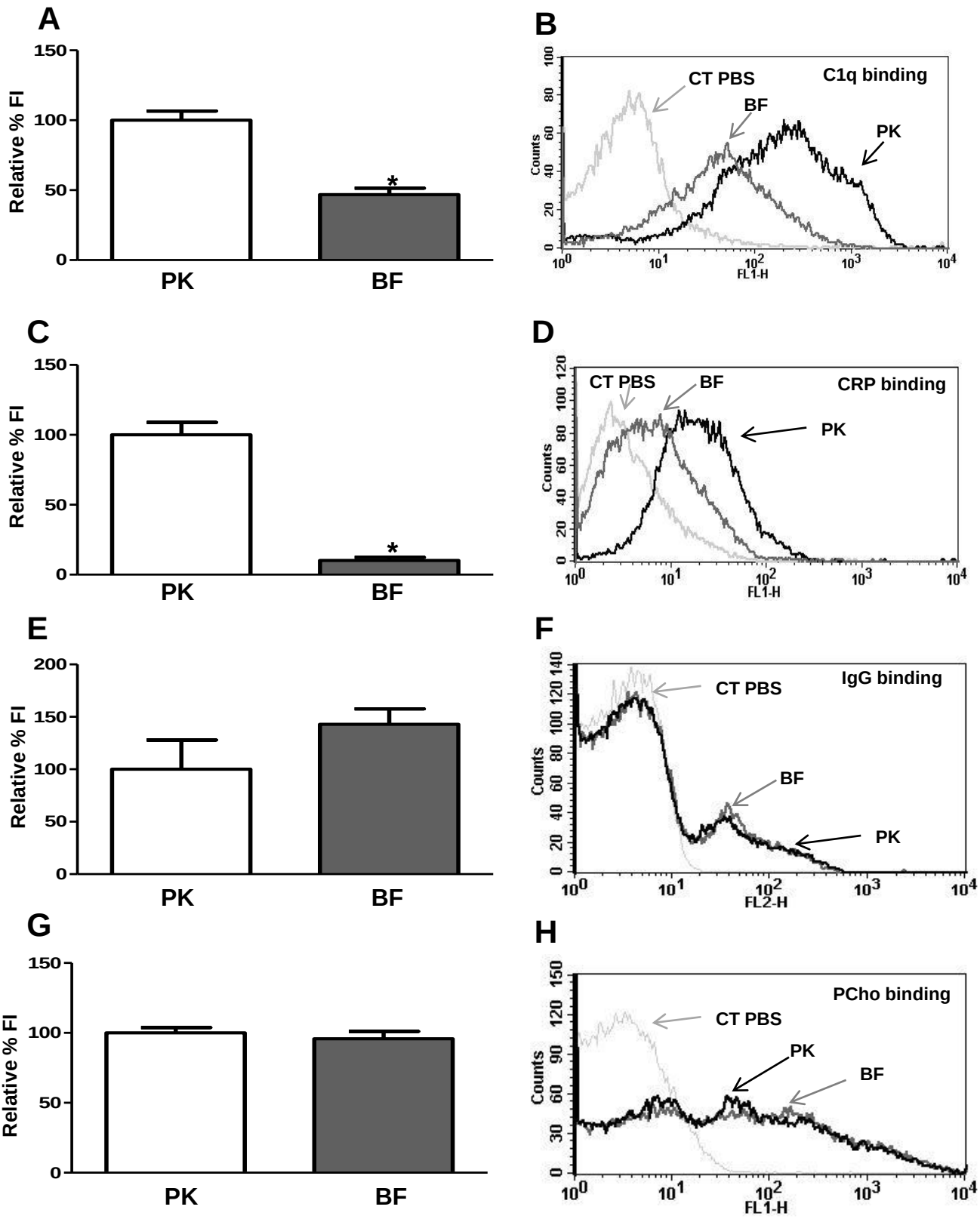

A
Relative % FI
150
100
50
0
*
PK
BF
B
CT PBS
C1q binding
BF
PK
Counts
FL1-H
C
Relative % FI
*
PK
BF
D
CT PBS
BF
CRP binding
PK
Counts
FL1-H
E
Relative % FI
200
150
100
50
0
PK
BF
F
CT PBS
IgG binding
BF
PK
Counts
FL2-H
G
Relative % FI
PK
BF
H
PCho binding
CT PBS
PK
BF
Counts
FL1-H

Figure 4

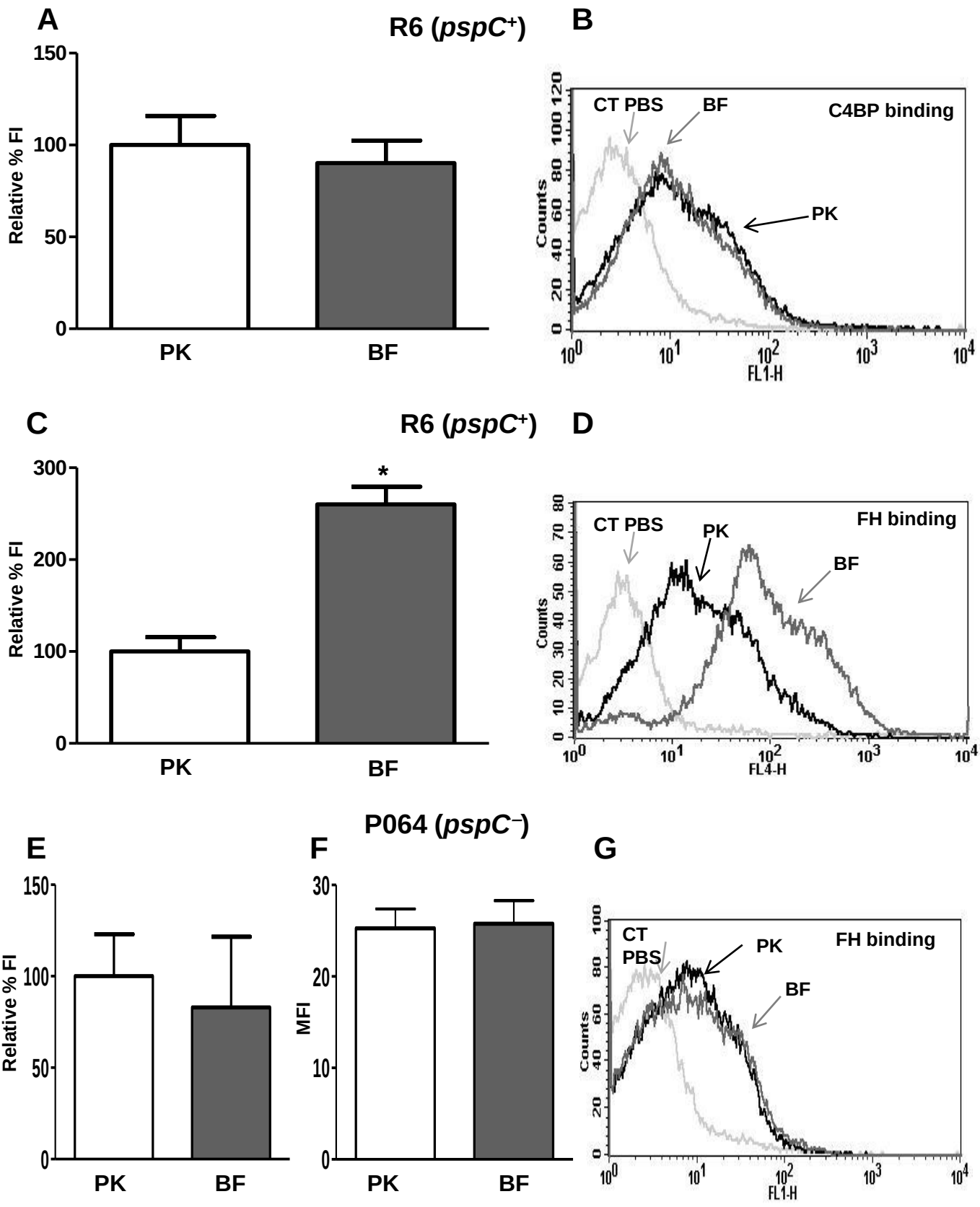
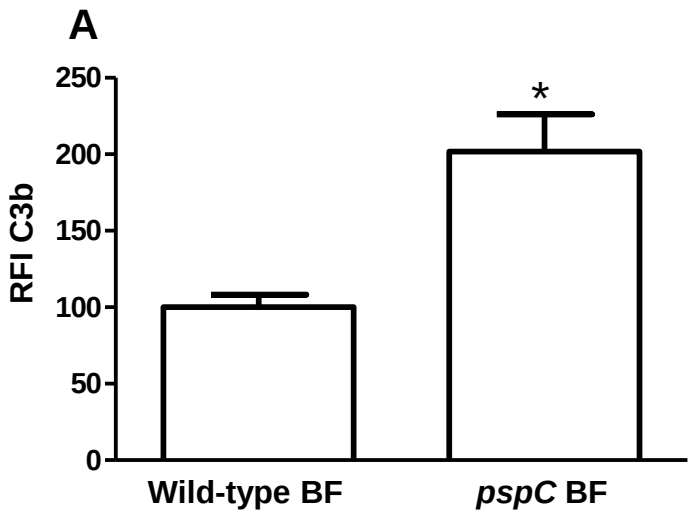
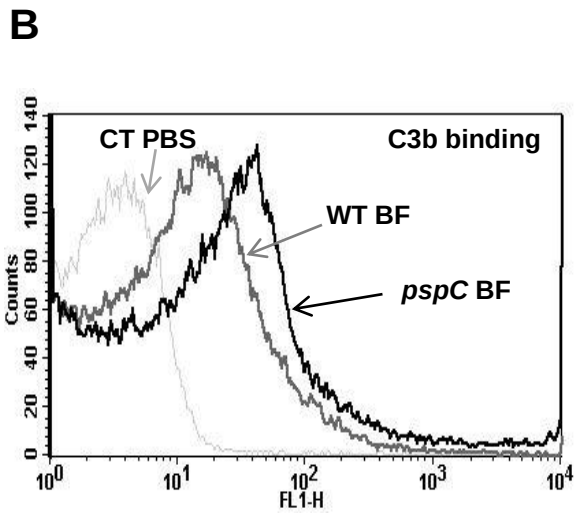
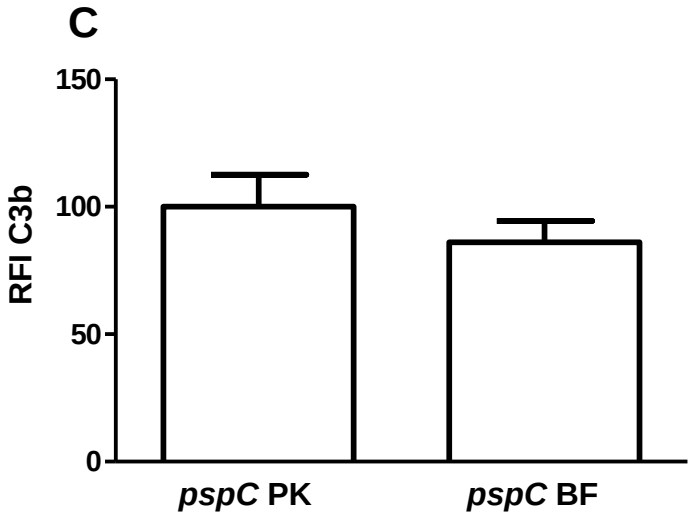

A
R6 (pspC+)
Relative % FI
150
100
50
0
PK
BF
B
CT PBS
BF
C4BP binding
PK
Counts
FL1-H
C
R6 (pspC+)
*
Relative % FI
300
200
100
0
PK
BF
D
CT PBS
PK
BF
FH binding
Counts
FL4-H
P064 (pspC–)
E
Relative % FI
150
100
50
0
PK
BF
F
MFI
30
20
10
0
PK
BF
G
CT
PBS
PK
BF
FH binding
Counts
FL1-H

Figure 5


A
RFI C3b
250
200
150
100
50
0
*
Wild-type BF
pspC BF


B
CT PBS
C3b binding
WT BF
pspC BF
Counts
FL1-H


C
RFI C3b
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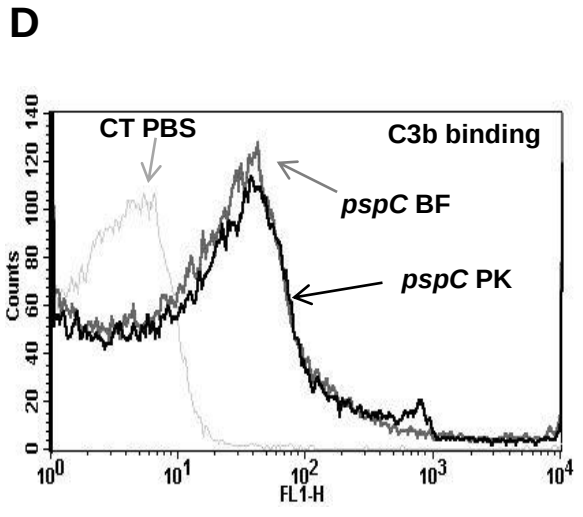
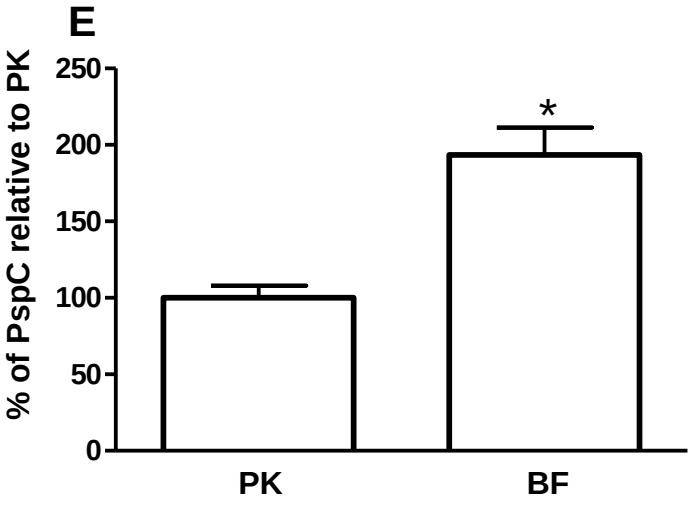
100
50
0
pspC PK
pspC BF


D
CT PBS
C3b binding
pspC BF
pspC PK
Counts
FL1-H


E
% of PspC relative to PK
250
200
150
100
50
0
*
PK
BF

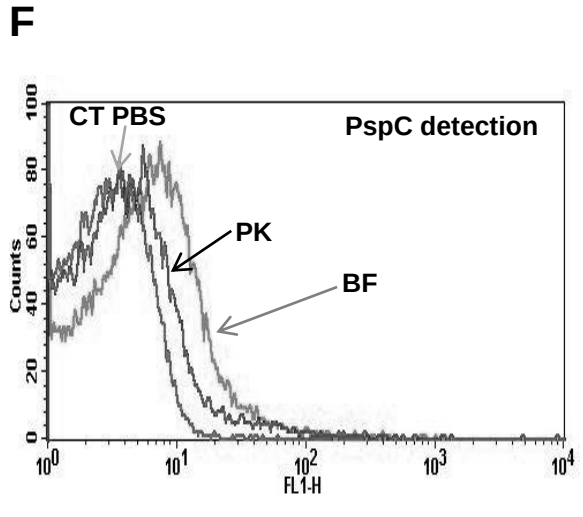
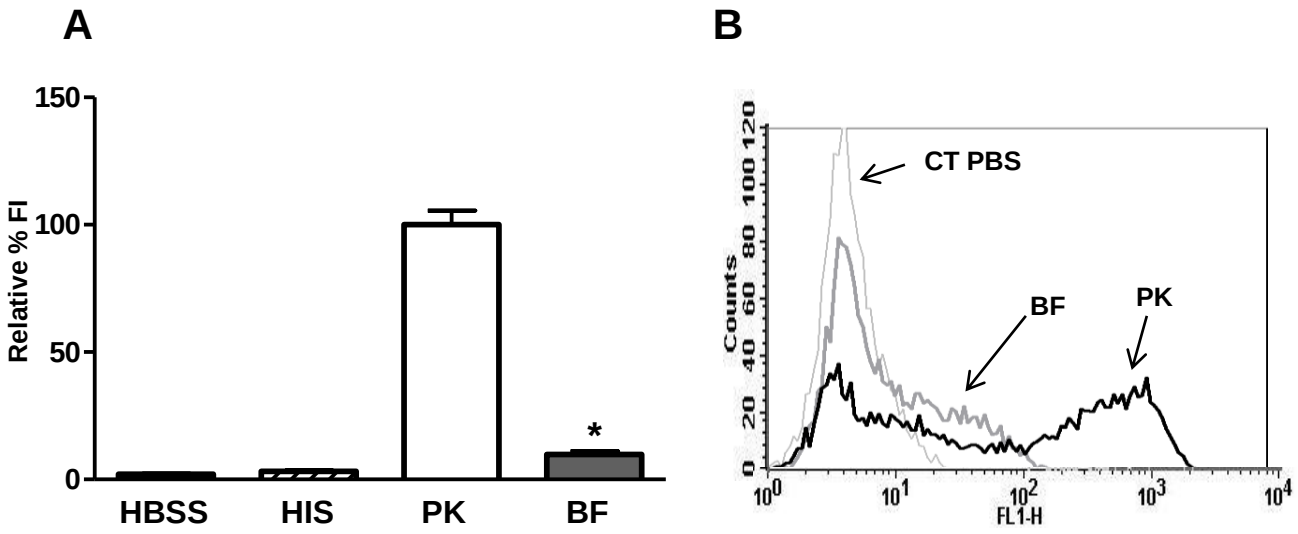

F
CT PBS
PspC detection
PK
BF
Counts
FL1-H

Figure 6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Relative % FI
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HBSS
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BF
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40
60
80
100
120
10^0
10^1
10^2
10^3
10^4
FL1-H