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The Virulence Function of Streptococcus pneumoniae Surface Protein A Involves Inhibition of Complement Activation and Impairment of Complement Receptor-Mediated Protection

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Complement is important for elimination of invasive microbes from the host, an action achieved largely through interaction of complement-decorated pathogens with various complement receptors (CR) on phagocytes. Pneumococcal surface protein A (PspA) has been shown to interfere with complement deposition onto pneumococci, but to date the impact of PspA on CR-mediated host defense is unknown. To gauge the contribution of CRs to host defense against pneumococci and to decipher the impact of PspA on CR-dependent host defense, wild-type C57BL/6J mice and mutant mice lacking CR types 1 and 2 (CR1/2−−), CR3 (CR3−−), or CR4 (CR4−−) were challenged with WU2, a PspA⁺ capsular type 3 pneumococcus, and its PspA− mutant JY1119. Pneumococci also were used to challenge factor D-deficient (FD−−) in infection with WU2. In comparison, PspA⁺/H11002 pneumococci have significantly reduced virulence compared with pneumococci that express PspA (PspA⁺) (2, 3). Earlier studies from our laboratory have shown that PspA inhibits complement activation, thereby limiting opsonization of pathogens by complement protein 3 (C3) (4–7). Ipso facto, PspA⁻ pneumococci that are avirulent in normal mice become virulent in C3-deficient (C3−−) and factor B-deficient (FB−−) mice (5).

It is currently accepted that three different pathways can lead to complement activation: the classical pathway, the mannose-bind- ing lectin (MBL) pathway, and the alternative pathway (8). All three pathways converge upon activation of C3, which is cleaved into C3a and C3b. This process exposes a thioester group on the α-chain of C3b that allows C3b to covalently attach to any activating surface in close proximity (9). With the assistance of various cofactors, surface-bound C3b is then sequentially processed, via the enzymatic activity of factor I, into smaller fragments (iC3b and C3d/C3dg). Importantly, C3b and its downstream cleavage products remain covalently bound to the target surface and act as ligands for different complement receptors (CRs) on various host phagocytes (10). Consequently, microbial pathogens sufficiently coated with C3 fragments are recognized by phagocytes.

The phagocyte CRs include CR1 (also called CD35), CR3 (also called αxβ2, Mac-1, or CD11b/CD18), and CR4 (also called αxβ2, p150,95, or CD11c/CD18) (11). CR1 reportedly has high affinity for C3b and lower affinity for iC3b, and also binds C4b, C1q, and MBL (12–14). Likewise, murine CR1 has binding sites for both C3b and C4b (15, 16). Because CR1 has cofactor activity, it also participates directly in complement activation by facilitating the factor I-mediated conversion of C3b to iC3b. This action of CR1 can down-regulate further complement activation (17). A closely related, but nonphagocytic, receptor, CR2 (CD21), binds C3dg/C3d and thereby influences B cell activation and development (16, 18). In the mouse, CR1 and CR2 are alternatively spliced products of a single Cr2 gene (19). In contrast to CR1, CR3 and CR4 have high affinity for iC3b and lower affinity for C3b. CR3 and CR4 are both members of the β2 integrin family, which also

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3 Abbreviations used in this paper: PspA, pneumococcal surface protein A; CR, complement receptor; FB, factor B; FD, factor D; MBL, mannose-binding lectin; WT, wild type.

Streptococcus pneumoniae is a major cause of bacterial pneumonia, otitis media, meningitis, and septicemia, especially in infants, the elderly, and immunocompromised individuals. The thick and rigid cell wall of the Gram-positive pneumococcus is protective against lysis by the complement membrane attack complex, so the main mechanism of complement-mediated elimination of pneumococci from infected hosts is by phagocytosis. Phagocytosis relies on the interaction of Ab and/or complement, deposited on the pathogen’s surface, with phagocyte receptors for the Fc portion of IgG or various complement proteins.

Pneumococcal surface protein A (PspA) is an important virulence factor for S. pneumoniae (1–3). Accordingly, in a murine model of bacteremia, PspA-deficient (PspA⁻) pneumococci have significantly reduced virulence compared with pneumococci that express PspA (PspA⁺) (2, 3). Earlier studies from our laboratory and others have shown that PspA inhibits complement activation, thereby limiting opsonization of pathogens by complement protein
FIGURE 1. Blood clearance/growth of PspA+ WU2 and PspA− JY1119 pneumococci in normal vs mutant mice. WT mice (solid lines) and mutant mice (dashed lines; deficient protein indicated) were infected i.v. with $\approx 2 \times 10^5$ CFU of WU2 (●) or JY1119 (○). Bacteremia was determined at different time points. Data are presented as the mean ± SEM CFU per milliliter of blood for six to 12 mice (indicated) in each group. The asterisks indicate that bacteremia in deficient mice is significantly different ($p < 0.05$) from that in WT mice infected with the same strain of pneumococcus.

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Materials and Methods

Animals

Wild-type (WT) C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Factor D-deficient (FD−/−), CD11b-deficient (CR3−/−), CD11c-deficient (CR4−/−), CD11a-deficient (LFA-1−/−), and CD18-deficient (CD18−/−; mice that lack functional CR3, CR4, and LFA-1) mice were from our own colonies. The construction and phenotype of each targeted mutant have been fully described previously (19, 21–23). All these mice are either C57BL/6J congenic or were backcrossed to C57BL/6J for at least six generations before being used. All animals were housed in separate cages, and none exhibited any evidence of infection before pneumococcal challenge at 10–12 wk of age.

Bacteria and infections

A capsular type 3 pneumococcal strain (WU2, PspA+) (24, 25) and its isogenic mutant (JY1119, PspA− ) (4, 7, 26) were used. Bacterial stocks were frozen at $-80^\circ$C in Todd-Hewitt broth supplemented with yeast extract until the OD$_{600}$ nm was 0.45 ($\approx 10^8$ CFU/ml), and 150 μl of bacterial culture was decanted. Bacteria were washed twice with PBS and resuspended in lactated Ringer’s buffer to achieve the desired working concentration. The number of bacteria injected into mice was confirmed by plating residual inoculums on blood agar. To assess the net clearance/growth of pneumococci in the circulation, mice were challenged i.v. with $2 \times 10^5$ CFU/mouse, and 50 μl of blood was collected from the retro-orbital plexus at 5 min, 1 h, 2 h, 4 h, and 6 h postinoculation. Blood was serially diluted and plated on blood agar plates to determine the number of viable S. pneumoniae recovered. The limit of detection was $10^2$ CFU of bacteria/ml blood; thus, when no pneumococci grew on culture plates, a level of bacteremia of $10^5$ CFU/ml was reported. The mortality of mice was monitored for 10 days after challenge.

Complement deposition assays

Pneumococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract until the OD$_{600}$ nm was 0.45 ($\approx 10^8$ CFU/ml), and 150 μl of bacterial culture was decanted. Bacteria were washed twice with PBS and resuspended in 50 μl of 10% normal mouse serum (isolated and pooled from five naive WT C57BL/6 mice) diluted in gelatin veronal buffer with calcium (0.15 mM) and magnesium (0.5 mM; GVB2). Pneumococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract containing 10% glycerol. To maintain the mutant PspA− phenotype, which carries an erythromycin resistance cassette, the culture medium for JY1119 always contained erythromycin (0.3 μg/ml). Frozen stocks of each strain containing a known concentration of viable bacteria were thawed and diluted in lactated Ringer’s buffer to achieve the desired working concentration.
Flow cytometry was used to further quantitate the amount of C3 deposited onto bacteria. Pneumococci were incubated with 30% FD−/− or WT serum in GVB+ at 37°C for 30 min. Bacteria incubated with only GVB+ served as a negative control. After washing, bacteria were stained with FITC-conjugated goat IgG to mouse C3, and the fluorescence intensity of each sample was determined using a FACScan instrument (BD Biosciences, Mountain View, CA).

**Surface expression of CRs on peripheral blood cells**

The expression of CR1/2 and CR3 on peripheral blood cells was determined by staining freshly isolated cells with fluorescent-conjugated goat anti-mouse CD21/35 or anti-mouse CD11b, respectively. Briefly, heparinized blood from WT, CR1/2−/−, and CD18−/− mice was washed with HBSS containing 0.1% BSA and stained with FITC-conjugated rat anti-mouse IgG to mouse C3, and the fluorescence intensity of each sample was determined using a FACScan instrument (BD Biosciences) to deplete erythrocytes. By flow cytometry, total leukocytes were gated based on forward and side scatter properties, and the fluorescence intensity profiles of the gated cell populations were determined.

**Measurement of serum Abs**

Sera from CD1/2−/−, CD18−/−, and WT mice were collected, and the levels of Abs directed against heat-killed pneumococci and phosphocholine Ag therein were determined by ELISAs. Microtiter plates were coated with heat-killed WU2 (10⁵ CFU/well in PBS) or phosphocholine-conjugated BSA (5 µg/well) overnight at 4°C. Mouse serum was serially diluted and added to the plates. ELISA plates were washed, sequentially developed with biotin-conjugated, goat anti-mouse Ig and streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL), and scanned at 405 nm.

**Statistical analysis**

To compare levels of bacteremia, Student’s t tests were applied to log-transformed data. To compare survival times (longevity) of WT and mutant mice infected with PspA+ and PspA− S. pneumoniae, Mann-Whitney U tests were used.

**Results**

**PspA+ pneumococci persist in the blood and cause fatal bacteremia in FD−/− mice**

We reported previously that when PspA+ vs PspA− pneumococci were used to challenge FB−/− mice, the PspA− bacteria were as virulent as PspA+ bacteria (5). However, because the mouse factor B-encoding gene, Bf, resides within the H-2 locus, we could not formally rule out the possibility that disruption of H-2, rather than absence of FB, was responsible for the increased susceptibility of FB−/− mice to PspA− pneumococci. FB and FD are both essential for formation of the alternative pathway C3 convertase. C3b, the activated form of C3, binds FB and presents it for cleavage by FD, yielding C3bBb, the alternative pathway C3 convertase. C3bBb sequentially activates more C3 and triggers the alternative pathway amplification loop. Consequently, like FB−/− mice, FD−/− mice have no operable alternative pathway and have impaired classical pathway activity due to the absence of an amplification loop. Importantly, FD−/− mice are not likely to have H-2 impairment.

We determined the ability of FD−/− to resist infection with the PspA+ and PspA− strains. The clearance/growth of pneumococci in WT and FD−/− mice was not different for the first 4 h after infection with PspA+ WU2, but pneumococcal burden was significantly higher (~5-fold higher) in FD−/− than WT by 6 h (Fig. 1A, ●). As a consequence, the longevity and survival rate of FD−/− mice challenged with WU2 were significantly lower than those in WT mice (p = 0.013; Fig. 2, left panel). In contrast, the PspA− JY1119 strain was completely cleared from the blood of

**FIGURE 2.** Survival times of WT and mutant mice infected i.v. with ~2 × 10⁵ CFU of WU2 (left panel) or JY1119 (right panel). Each dot or cross represents the death of a single mouse, and the mutant gene carried by mice is indicated on the abscissa. For animals infected with each kind of bacteria, the median time to death (horizontal lines) is shown. The longevity of mutants was compared with that of WT mice, and mutants whose survival time was significantly less than that of WT mice infected with the same bacteria (p < 0.05) are indicated with an asterisk.
20% (one of five) of CR1/2 WT mice challenged with JY1119 survived infection, but only clearance/killing of blood-borne PspA panel. These data reveal that, like FB and FD, CR1/2 is required for WT mice; bacteremia was fairly constant throughout the experiment. Thus, at 4 h postinfection, 10-fold more PspA pneumococci were recovered from the blood of CR1/2 mice than from contemporaneous samples from WT mice. In WU2-infected CR1/2 mice, the 10-day survival rate (12%; one of eight) was lower than that in WU2-infected WT (42%; five of 12), although median longevity was not significantly different. All WT mice challenged with JY1119 survived infection, but only 20% (one of five) of CR1/2 mice died (p = 0.018; Fig. 2, right panel). These data reveal that, like FB and FD, CR1/2 is required for full protection from pneumococci. Clearance/killing of blood-borne PspA pneumococci in CR3−/− or CR4−/− mice is slower, but does not lead to fatal infection. The residence time of WU2 in the bloodstream of CR3−/− and CR4−/− mice was not substantially different from that observed in WT mice; bacteremia was fairly constant throughout the experiment in all cases (Fig. 1, C and D). Despite this, both kinds of receptor-deficient mice showed increased death rates; 88% of CR3−/− and 75% of CR4−/− died within 72 h compared with 58% of WT mice (Fig. 2, left panel). The median longevity of CR3−/− and CR4−/− mice was significantly reduced compared with that of WT mice (p = 0.003 for CR3−/−; p = 0.007 for CR4−/−). In contrast to WU2, clearance/killing of JY1119 was significantly slower in both CR3−/− and CR4−/− compared with WT mice; thus, at 4 h postinfection, 100-fold more PspA pneumococci were recovered from CR3−/− than WT mice (Fig. 1C), and 10-fold more was recovered from CR3−/− (Fig. 1D). However, unlike CR1/2−/− mice, all CR3−/− and all CR4−/− mice survived infection with JY1119. These data indicate that CR3 and CR4 also are required for full protection from pneumococci.

Clearance/killing of blood-borne pneumococci is slower in CR1/2−/− mice, leading to fatal infection

For up to 6 h postinfection, no significant difference in blood clearance/killing of WU2 was observed in CR1/2−/− mice compared with WT mice (Fig. 1B, ○); however, the clearance of JY1119 was significantly delayed in CR1/2−/− compared with WT mice (Fig. 1B, ○). Thus, at 4 h postinfection, 10-fold more PspA pneumococci were recovered from the blood of CR1/2−/− mice than from contemporaneous samples from WT mice. In WU2-infected CR1/2−/− mice, clearance/killing of WU2 was observed in CR1/2−/− mice (Fig. 2, left panel). Together, these results reinforce those we reported previously using FB−/− mice, confirming the importance of an intact alternative pathway for the efficient clearance/killing of pneumococci in the mouse. They also support our hypothesis that PspA circumvents the role of this pathway in protection.

Low expression of CR1/2 and high anti-pneumococcal Ab titers in CD18−/− mice

CD18 is the common β-chain shared by CR3, CR4, and LFA-1; thus, deficiency of CD18 results in a lack of all three functional molecules; yet CD18−/− mice resisted infection. A compensatory increase in the expression of CR1/2 in CD18−/− mice could explain this seeming paradox. However, CD18−/− mice had >2-fold less expression of CR1/2 on their PBL than WT mice (Fig. 4A, compare bottom left and bottom right panels). Alternatively, a compensatory heightened level of naturally occurring Abs in CD18−/− could also explain our observation. Indeed, we observed that CD18−/− did have much higher levels of IgG reactive with heat-killed pneumococci (Fig. 4B, left panel). It is therefore likely that PspA circumvents the role of this pathway in protection.
respectively, but by 60 min, the advantage was negated. The difference in the amount of iC3b deposited onto the two strains of pneumococci was more obvious when mouse, rather than human, serum was used as the complement source, but the reaction was slower. These findings are consistent with the proposal that more C3 is deposited on JY1119 than WU2, the majority of C3 deposited is iC3b, and the rate of conversion of C3b to iC3b is increased on JY1119. The fact that similar observations were made using mouse vs human serum suggests that this has clinical relevance.

Discussion

In this study and previous ones (4–7), we showed that substantially less C3 is deposited onto the PspA\textsuperscript{+} \textit{S. pneumoniae} strain WU2 than onto its isogenic PspA\textsuperscript{−} counterpart JY1119 regardless of the serum source, and that WT mouse serum supports more deposition of C3 onto the pneumococcal surface than FB\textsuperscript{−}/H11002 (7) or FD\textsuperscript{−}/H11002 (53) serum (Fig. 3). This decrease in deposition of C3 in vitro correlates with impaired host resistance to infection. Thus, in FD\textsuperscript{−}/H11002 mice (Figs. 1 and 2), JY1119 becomes as virulent and as lethal as WU2 is in normal mice. Together with observations made previously by others (27), our initial findings (5), and the new data we report in this study clearly indicate that in mice an intact alternative pathway is vital for proper host defense against \textit{S. pneumoniae} infection, and that PspA impairs this pathway of protection. Recently, Brown et al. (28) argued that the classical pathway is the dominant pathway for host innate immunity against pneumococcal infection in mice. Our current findings are not at odds with this proposition. Indeed, the classical and alternative pathways are probably both required for full protection against pneumococcal infection, because the latter pathway amplifies C3 deposition initiated by the former.

Immunoblots provided indirect evidence that one of the major forms of C3 on opsonized U2 and JY1119 is iC3b, a fragment known to serve as an efficient ligand for CR3, CR4, and perhaps also CR1/2. For mice infected with U2, the strain on which C3 deposition and blood clearance/killing is limited by the presence of PspA, deficiency of any one of these CRs is thus sufficient to increase the mortality rates of mice. Notably, the negative impact on survival rate and longevity achieves statistical significance in CR3\textsuperscript{−}/H11002 and CR4\textsuperscript{−}/H11002 mice, an outcome consistent with the reported high affinity binding of iC3b to these receptors. In contrast, in mice challenged with JY1119, to which more C3 binds and blood clearance/killing is more efficient because PspA is absent, deficiency of CR3 or CR4 did not impact survival, but deficiency of CR1/2 significantly decreased it. Furthermore, CR1/2\textsuperscript{−}/H11002, CR3\textsuperscript{−}/H11002, and CR4\textsuperscript{−}/H11002 mice all showed retarded removal of JY1119 from the bloodstream.

CR1/2 also plays an important role in the humoral immune response. Haas et al. (19) reported that serum IgM, IgG1, IgG2b, and IgG3 are all significantly reduced in CR1/2\textsuperscript{−}/H11002 mice, whereas IgG2a and IgA are normal. In our hands, the titer of anti-pneumococcal Abs in CR1/2\textsuperscript{−}/H11002 did not differ from WT mice, whereas the titer of anti-phosphocholine Abs was indeed substantially lower. Anti-phosphocholine Abs are known to be protective against pneumococcal infection (29, 30); thus, CR1/2\textsuperscript{−}/H11002 mice are probably highly susceptible to the normally avirulent PspA\textsuperscript{−} pneumococci, because, on the one hand, they lack CR1/2 and, on the
other, they have reduced titers of this (and perhaps other) protective Abs. CR1 probably also interacts with C1q and MBL (12–14), and CR1 and Fc receptors exert synergetic effects to enhance phagocytosis (31), so the host defense role of CR1/2 in mice infected with JY1119 probably has additional complexity and importance. The relative importance of factor H-mediated clearance of opsonized bacteria by platelets, which presumably direct uptake of the organism by the liver and spleen (32), also remains to be determined.

Previous studies showed that CD18-deficient animals have increased spontaneous infections and earlier mortality compared with WT mice (33). Others reported that LFA-1/H11002/H11002 mice have increased mortality and a higher incidence of otitis media. These mice are more likely to develop meningitis after being challenged with S. pneumoniae i.p., and the LFA-1-ligand interaction is the stimulatory signal for neutrophils to express full activation. In our hands, there was no significant difference in mortality between CR3/H11002/H11002, CR4/H11002/H11002, and CD18/H11002/H11002 mice after infection with either PspA+/+ or PspA− pneumococci. The fact that CR3−/− and CR4−/− mice express normal amounts of CR1/2 (data not shown) partially explains why these animals are still resistant to JY1119. LFA1−/− and CD18−/− mice cleared WU2 somewhat more rapidly than WT, and the survival time of WU2- and JY1119-infected animals was not adversely affected by deficiency of LFA-1. This difference between our observations and those of others might be due to the different outcome parameters and routes of infection used; i.e., we focused on blood clearance/killing after i.v. infection, whereas others used an i.p. infection model to emphasize the migration ability of phagocytes into the sites of infection. Moreover, in an earlier i.p. experiment a serotype 6 pneumococcal strain was used (34), whereas we used a serotype 3 strain. Notably, LFA-1−/− animals reportedly have a higher number of neutrophils in the peripheral blood than WT (33, 34), which could also account for their improved resistance to i.v. infection.

The seeming paradox that CD18−/− mice remain fully resistant can be explained. CD18−/− mice lack CR3 and CR4 and have
lower expression of CR1/2, which tends to make them more susceptible. In contrast, these mice are predicted to have neutrophilia associated with their lack of LFA-1 (33, 34), and we showed that they express elevated levels of anti-pneumococcal Abs. We propose that in CD18−/− mice, the combined benefits of neutrophilia and high protective Ab titers overcomes the detrimental effects of low CR1/2 expression and the absence of CR3 and CR4.

As in this study, others have infected mutant mice with S. pneumoniae to study the contributions to protection made by CR1/2, CR3, CD18, and LFA-1 (19, 33, 34). These earlier studies focused on the impact of CR1/2 deficiency on the development of protective adaptive immunity or on the effects of CD18, LFA-1, and CR3 on long term survival after i.p. inoculation of pneumococci. Notably, our study is the first comprehensive investigation of the contribution of these receptors to protection against PspA+ and PspA− pneumococcal strains and to date is the only one to study the impact of CR4 deletion on host defense. In summation, we conclude that PspA confers virulence on S. pneumoniae mainly by interfering with the protective role of the alternative pathway of complement activation, thus minimizing C3b deposition and slowing iC3b generation. The pneumococcus is thereby shielded from host CR-mediated pathways of host defense.

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References


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