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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

Bing Ren,* Mark A. McCrory,† Christina Pass,‡ Daniel C. Bullard,§ Christie M. Ballantyne,§ Yuanyuan Xu,‡ David E. Briles,* and Alexander J. Szalai‡‡

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 pneumoccus, and its PspA− mutant


**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 IgS 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 3 (C3) (4–7). Importantly, 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-
A capsular type 3 pneumococcal strain (WU2, PspA\(^{+}\)/H11001) and its isogenic mutant (JY1119, PspA\(^{−}\)/H11002) were used. Bacteria were washed twice with PBS and resuspended in 50 \(\mu\)l of 10% normal mouse serum (isolated and pooled from five naive WT C57BL/6J mice) diluted in gelatin veronal buffer with calcium (0.15 mM) and magnesium (0.5 mM; GVB2, Sigma-Aldrich, St. Louis, MO). The bacteria were thus opsonized at 37°C for 5, 10, or 30 min, after which ice-cold PBS buffer containing 10 mM EDTA was added to stop the reaction. Opsonized bacteria were washed, and the bacteria were resuspended in 50 \(\mu\)l of reducing SDS sample buffer, boiled for 10 min, and loaded onto SDS-polyacrylamide gels for electrophoresis. Subsequent immunoblots were prepared and developed using goat anti-mouse C3 polyclera (Bethyl Laboratories, Montgomery, TX) in concert with biotin-conjugated streptavidin. Immunoreactive bands of protein were quantitated by densitometry using the Bio-Rad gel documentation system (Bio-Rad, Hercules, CA). To validate the findings and determine their clinical relevance, the experiment was repeated using normal human serum (lot OM1013; Quidel, San Diego, CA). In this case, purified human C3, C3b, and iC3b proteins were used as controls, and the immunoblot was developed using goat anti-human C3 polyclera (both from Quidel).
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 fluorochrome-conjugated goat anti-mouse CD21/35 or R-PE-conjugated mAb M1/70 (anti-CD11b), both obtained from BD Pharmingen (San Diego, CA). After one wash, each blood sample was incubated with FACS lysing solution (BD Biosciences, Mountain View, CA) 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 (10^8 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 × 10^8 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.](http://www.jimmunol.org/content/7508/2/808/F2.large.jpg)
WT in <4 h, but persisted in FD−/− (Fig. 1A, ○). WT mice challenged with JY1119 all survived at least 10 days, whereas FD−/− mice challenged with JY1119 had significantly shortened longevity (3.5 days; p = 0.004 compared with WT) and only 14% survived (Fig. 2, right panel). In vitro, WT serum supported more C3 deposition onto pneumococci than did FD−/− serum regardless of whether WU2 or JY1119 was being opsonized, and the amount of C3 deposited onto JY1119 was consistently greater than that deposited onto WU2 (Fig. 3). 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.

**Clearance/killing of blood-borne PspA− 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, 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 (Fig. 2, left panel). 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 marginally improved in LFA-1−/− and CD18−/− mice

Both PspA+ and PspA− bacteria disappeared from the blood of LFA-1−/− marginally faster than from WT mice (Fig. 1E, dashed lines), and the longevity of WU2-infected LFA-1−/− mice was somewhat prolonged (Fig. 2, left panel). Similarly, and despite their inherited absence of functional CR3 and CR4, WU2 was removed more quickly from the blood of CD18−/− than from WT mice (Fig. 1F, dashed lines), and no difference was observed in the survival time of CD18−/− and WT mice infected with either pneumococcus (Fig. 2).

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 bacteremia is lessened, and survival rate is unaffected in CD18−/− mice compared with WT mice, because they make higher than normal levels of protective anti-pneumococcal Abs. Likewise, although CR1/2−/− mice had normal expression of CR3 (Fig. 4A) and WT-like levels of anti-pneumococcal Abs (Fig. 4B, lower right panel), their lower levels of anti-phosphocholine Abs (Fig. 4B, lower right panel) might account for their extreme susceptibility.

Degradation of C3 on the pneumococcal surface

Because CR1/2, CR3, and CR4 have variable affinities for similar C3-derived ligands, we sought to determine which C3-derived fragments are present on the surface of serum-opsonized pneumococci. To this end, WU2 and JY1119 were incubated in 10% normal serum to allow opsonization to proceed. The incubation was terminated at different time points by the addition of cold PBS buffer containing EDTA. C3 retained on the surface of the opsonized bacteria was then revealed by immunoblot analysis (Fig. 5A). Based on comparison with purified human C3, C3b, and iC3b controls and the presence of the α2 fragment (Fig. 5B), the major form of C3 deposited on WU2 and JY1119 appears to be iC3b, although C3b was also present in some samples. As incubation time was increased, iC3b accumulated on both types of bacteria (Fig. 5A). Importantly, however, at each time point comparatively more iC3b was present on JY1119 than on WU2. With the Bio-Rad gel documentation system, we measured the densities of α2 and β subunits in each sample. At 5, 30, and 60 min, the β-chain signal was 1.4-, 1.47-, and 1.32-fold greater, respectively, for JY1119 than for WU2. This indicates that deposition of C3 per se was consistently greater for the PspA− strain. Contemporaneously, the α2 fragment was 2.03-, 1.91-, and 1.09-fold more intense, respectively, for JY1119 than for WU2. This indicates that processing of deposited C3 is more rapid on JY1119 than on WU2. Based on the α2/β ratio, processing of C3b to iC3b was estimated to be 1.4- and 1.3-fold greater for JY1119 than WU2 at 5 and 30 min.

**FIGURE 3.** C3 deposition on pneumococci in the presence of WT and FD−/− mouse sera. Pneumococci were incubated in 30% pooled WT or FD−/− serum at 37°C for 30 min, and C3 deposition was detected by flow cytometry using FITC-conjugated goat anti-mouse C3 IgG. For WU2 incubated with WT serum, FD−/− serum, and buffer (no serum), the mean fluorescence intensities achieved were 71.2, 36.8, and 4.1 U, respectively. For JY1119 incubated with WT serum, FD−/− serum, or buffer, the mean fluorescence intensities were 168.9, 64.5, and 3.6, respectively.
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\(^+\) *S. pneumoniae* strain WU2 than onto its isogenic PspA\(^-\) counterpart JY1119 regardless of the serum source, and that WT mouse serum supports more deposition of C3 onto the pneumococcal surface than FB\(^+\) or FD\(^+\) serum (Fig. 3). This decrease in deposition of C3 in vitro correlates with impaired host resistance to infection. Thus, in FD\(^+\) 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 *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 WU2 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 WU2, 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\(^-\) and CR4\(^-\) 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, CR3\(^-\), and CR4\(^-\) 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\(^-\) mice, whereas IgG2a and IgA are normal. In our hands, the titer of anti-pneumococcal Abs in CR1/2\(^-\) 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\(^-\) mice are probably highly susceptible to the normally avirulent PspA\(^-\) 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 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, CR4/H11002, and CD18/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

**FIGURE 5.** A, Western blot analysis of C3 deposition on PspA+ (WU2) and PspA− JY1119 (JY) pneumococci. A, Pneumococci were opsonized with human serum (left panel) or mouse serum (right panel) for the times indicated, washed with PBS, boiled in reducing buffer, and electrophoresed, then transferred to nitrocellulose. Membranes were developed with anti-human or anti-mouse C3 Abs as required. Purified human (Hu) C3, C3b, and iC3b were included as controls. The identity of each chain or degraded fragment of C3 is indicated. B, Schematic view of C3 processing during complement activation. After covalent attachment of C3b to pneumococci via the α-chain thioester bond (*), cleavage of the α-chain of C3 by the cofactor-dependent action of factor I (FI) generates iC3b, which remains bound to the opsonized bacterium.
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 onto 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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