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Enhanced Susceptibility to Acute Pneumococcal Otitis Media in Mice Deficient in Complement C1qa, Factor B, and Factor B/C2[▽]

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To define the roles of specific complement activation pathways in host defense against *Streptococcus pneumoniae* in acute otitis media (AOM), we investigated the susceptibility to AOM in mice deficient in complement factor B and C2 (*Bf*/*C2*$^{-/-}$), C1qa (*C1qa*$^{-/-}$), and factor B (*Bf*$^{-/-}$). Bacterial titers of both *S. pneumoniae* serotype 6A and 14 in the middle ear lavage fluid samples from *Bf*/*C2*$^{-/-}$, *Bf*$^{-/-}$, and *C1qa*$^{-/-}$ mice were significantly higher than in samples from wild-type mice 24 h after transtympanical infection ($P < 0.05$) and remained persistently higher in samples from *Bf*/*C2*$^{-/-}$ mice than in samples from wild-type mice. Bacteremia occurred in *Bf*/*C2*$^{-/-}$, *Bf*$^{-/-}$, and *C1qa*$^{-/-}$ mice infected with both strains, but not in wild-type mice. Recruitment of inflammatory cells was paralleled by enhanced production of inflammatory mediators in the middle ear lavage samples from *Bf*/*C2*$^{-/-}$ mice. C3b deposition on both strains was greatest for sera obtained from wild-type mice, followed by *C1qa*$^{-/-}$ and *Bf*$^{-/-}$ mice, and least for *Bf*/*C2*$^{-/-}$ mice. Opsonophagocytosis and whole-blood killing capacity of both strains were significantly decreased in the presence of sera or whole blood from complement-deficient mice compared to wild-type mice. These findings indicate that both the classical and alternative complement pathways are critical for middle ear immune defense against *S. pneumoniae*. The reduced capacity of complement-mediated opsonization and phagocytosis in the complement-deficient mice appears to be responsible for the impaired clearance of *S. pneumoniae* from the middle ear and dissemination to the bloodstream during AOM.

Streptococcus pneumoniae is one of the major bacterial pathogens that cause childhood otitis media (OM). It accounts for 30% of cases of acute OM (AOM) and 5% of chronic OM with effusion (OME) (10). Chronic nonsuppurative OME is generally considered to be benign and self-limiting, but persistent accumulation of fluid in the middle ear space is associated with hearing loss. This is detrimental to language development and learning during early childhood development (8). Despite recent advances in our understanding of the pathogenesis of *S. pneumoniae* OM, more needs to be learned about the protective role of the host innate immune defense systems during pneumococcal OM.

The complement system is a major component of the host innate immune defense system against infection (18). Activation of the complement system in response to invading pathogens is mediated through the classical, alternative, and lectin pathways. Activation results in C3 cleavage, releasing anaphylatoxins C3a and C5a, and formation of a membrane attack complex to lyse the target cells. Cleavage of C3 generates the key opsonins, C3b and iC3b. These proteins tag the pathogens for phagocytosis. The classical complement pathway involves C1, C2, C4, and C3. It is activated by C1q usually in response to formation of antigen-antibody immune complexes, but it can also be activated by bacterial cell walls, C-reactive protein, and serum amyloid P component. Mice that do not express C1q are more likely to die when infected intranasally with *S. pneumoniae* (4). The alternative pathway involves C3, factor B, factor D, and properdin and can be activated by microbial fragments. Spontaneously hydrolyzed C3 complexes with factor B. Factor B is then cleaved by factor D to generate C3bBb, the alternative pathway C3 convertase. Cleavage of C3 by the alternative pathway C3 convertase can deposit C3b onto bacterial surfaces. C3b generated from the classical/mannose-binding lectin (MLB) pathway can also directly bind factor B and form the alternative pathway convertase, which in turn activates more C3 and generates more C3b. It has been shown that the alternative pathway plays an amplification role for complement activation of the classical/MBL pathway leading to denser C3 deposition on *S. pneumoniae* (4). The lectin pathway is initiated by the binding of MBL or ficolins to carbohydrate groups on the pathogen's surface and appears to play a minor role in complement activation by *S. pneumoniae* (4).

It has been suggested that both the classical and alternative complement pathways are of central importance in host defense against *S. pneumoniae* in mouse models of pneumonia and systemic infection (4). C1qa-deficient (*C1qa*$^{-/-}$) mice (which are unable to activate complement through the classical pathway) have been found to be more susceptible than factor B-deficient (*Bf*$^{-/-}$) mice (which are unable to activate complement through the alternative pathway) to pneumococcal infection (4). This supports the concept that the classical path-

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way is the dominant mechanism for the innate immune response, at least in these models (4).

Clinical observations and the results of experimental studies suggest that the complement system plays a significant role in middle ear defense against OM. Components of the classical and alternative pathways have long been identified in middle ear effusion samples from OME patients (15). However, 73% of middle ear effusion samples from young patients showed no immunoglobulin- or C3b-coating bacteria, including *S. pneumoniae* (22). This indicates that pathogens in the middle ear cavities were not opsonized and prepared for phagocytosis by neutrophils and macrophages (22). A recent experimental study has shown that decomplementation by cobra venom factor (CoVF) increases the virulence of high C3 binding *S. pneumoniae* in the chinchilla OM model (19). The relative contribution of each of the specific complement pathways and complement cascades in protection against *S. pneumoniae* OM remains largely unknown. To examine this issue in greater detail, we transtympanically inoculated the middle ears of wild-type and complement-deficient *C1qa*$^{-/-}$, *Bf*$^{-/-}$, and *Bf*/*C2*$^{-/-}$ mice with *S. pneumoniae* and evaluated the course of pneumococcal AOM in each strain of mice. We found that both the classical and alternative pathways are critical components of the otological innate immune defense against *S. pneumoniae*. We were also able to show that complement-deficient mice have a reduced capacity to elicit complement-mediated opsonization and opsonophagocytosis. The reduced capacity of complement-mediated opsonization and phagocytosis in *Bf*/*C2*$^{-/-}$ mice and to a lesser extent in other complement-deficient mice appears to be responsible for the impaired clearance of *S. pneumoniae* from the middle ear and dissemination to the bloodstream during the early stages of AOM.

MATERIALS AND METHODS

Bacteria. *S. pneumoniae* type 6A (EF3114) with a predominant transparent phenotype was provided by B. Anderson, Department of Clinical Immunology, University of Göteborg, Sweden. *S. pneumoniae* type 14 with a uniformly transparent phenotype was obtained from the Centers for Disease Control and Prevention, Atlanta, GA. Both strains have been used for OM research in our laboratories and have previously been described in detail (1, 24, 25). Log-phase cultures were prepared by inoculating Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI) with *S. pneumoniae* grown overnight on Columbia CNA agar plate. After 3-h incubation, the cultures were centrifuged at 3,500 × *g* for 20 min, washed twice, and resuspended in sterile pyrogen-free saline as previously described (24). The concentration of *S. pneumoniae* (in CFU per milliliter) was determined by standard dilution and plate count.

Mice and mouse sera. Eight- to 12-week-old male or female C57BL/6 mice were used for this study. C57BL/6 mice homozygous for combined gene deficiencies of factor B and C2 (*Bf*/*C2*$^{-/-}$) or C1qa (*C1qa*$^{-/-}$) were generated as described previously (2, 23), and breeding colonies of *Bf*/*C2*$^{-/-}$ and *C1qa*$^{-/-}$ mice were maintained at Taconic Farms (Germantown, NY). C57BL/6 mice homozygous for a gene deficiency of factor B (*Bf*$^{-/-}$) were obtained from the University of Colorado at Denver and Health Sciences Center (12). *C1qa*$^{-/-}$, *Bf*/*C2*$^{-/-}$, and *Bf*$^{-/-}$ mice were backcrossed at least 9 generations onto the C57BL/6 background. Age- and sex-matched C57BL/6 mice used as controls were from Taconic Farms. Blood samples from 3 to 5 mice with the same genetic background were obtained by cardiac puncture. Single use aliquots of the sera were stored at −70°C. All study procedures were approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Mouse model of AOM. Mice were anesthetized by intraperitoneal injection with ketamine hydrochloride (20 mg/kg of body weight) and xylazine (5 mg/kg). AOM was then produced by direct bilateral transtympanical inoculation of the middle ear. The inoculum consisted of 5 μl of a suspension containing approximately 1 × 10^3 CFU of *S. pneumoniae* in sterile pyrogen-free saline as previously described (11, 21). Cohorts of 6 to 11 mice were used at each time point. At 4, 24, 48, and 72 h postinoculation, mice were anesthetized and then sacrificed. The middle ear spaces were lavaged to quantitatively determine the titers of *S. pneumoniae* and inflammatory cells. The middle ear space was rinsed four times with 5 μl of sterile pyrogen-free saline, and the washings were aspirated and pooled. The inflammatory cell concentration of each sample was determined with a hemocytometer. Blood was collected via cardiac puncture and quantitatively cultured to determine bacterial dissemination. The middle ear lavage and blood samples were cultured overnight at 37°C on Columbia CNA agar plates in an incubator supplemented with humidity and 5% CO_2. The number of CFU per milliliter was determined by a standard dilution assay and plate counting.

Histology. Six temporal bones from each cohort were removed immediately after sacrifice at 24, 48 and 72 h postchallenge. The samples were processed as described previously with minor modifications (11). The temporal bones were fixed in 10% neutral-buffered formalin and decalcified with EDTA. The specimens were further processed for conventional paraffin embedding. Serial sections were cut to a thickness of 6 μm and stained with hematoxylin and eosin (H&E).

Analysis of C3b deposition on *S. pneumoniae* by flow cytometry. Freshly prepared 5 × 10^7 CFU of *S. pneumoniae* were incubated with neat mouse sera from wild-type, *C1qa*$^{-/-}$, *Bf*$^{-/-}$, and *Bf*/*C2*$^{-/-}$ mice for 30 min at 37°C as previously described (4). The bacteria were washed with Dulbecco's phosphate-buffered saline (DPBS; pH 7.2), resuspended in 50 μl of DPBS–0.1% Tween 20 containing a 1:300 (vol/vol) dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse C3 antibody (MP Biomedicals, Aurora, OH), and then incubated for 30 min on ice in the dark. The bacteria were then washed and resuspended in 1% paraformaldehyde in DPBS. C3b deposition was detected using a FACSCalibur (BD Biosciences, San Jose, CA). Bacteria incubated with DPBS were used as negative controls to set the threshold and fluorescence intensity. A minimum of 20,000 cells per sample were analyzed. The data are expressed as the mean ± standard deviation of the proportion of bacteria showing fluorescence. Each assay was performed at least three times using different lots of sera.

C3b deposition on *S. pneumoniae* recovered from middle ear lavage fluid and blood samples at 24 h postchallenge was determined as previously described with minor modifications (16). Briefly, the middle ear lavage and blood samples pooled from two mice in each cohort were centrifuged at 800 × *g* for 5 min at 4°C. The supernatants were centrifuged at 15,500 × *g* for 10 min at 4°C, and the bacterial pellets were washed once in DPBS and incubated with FITC-conjugated anti-mouse C3 antibody. The experiment was repeated three times.

Opsonophagocytosis. Opsonophagocytosis was determined as previously described (27). Briefly, *S. pneumoniae* bacteria were fluorescently labeled by incubation with 5,6-carboxyfluorescein-succinimidyl ester (FAM-SE; Molecular Probes) solution (10 mg/ml in dimethyl sulfoxide; Sigma) in 0.1 M sodium bicarbonate buffer for 1 h at 37°C. They were washed six times with Hanks balanced salt solution (HBSS) containing 0.2% bovine serum albumin (BSA) and stored in aliquots at −70°C in 10% glycerol (approximately 10^9 CFU/ml). The human tissue culture cell line HL-60 was used. HL-60 cells (promyelocytic leukemia cells) (CCL-240; American Type Culture Collection) were differentiated into granulocytes according to the laboratory protocol for "*Streptococcus pneumoniae* opsonophagocytosis using differentiated HL-60 cells" that is available from the website http://www.vaccine.uab.edu. FAM-SE-labeled bacteria (10^6 CFU) were opsonized with 10 μl of 10% mouse serum for 20 min at 37°C. HL60 cells (10^5) were added to the opsonized bacteria and incubated for 30 min at 37°C, after which the bacteria and cells were fixed using 3% paraformaldehyde and analyzed using a FACSCalibur to assess the proportion of cells associated with bacteria. Negative controls were included, using the same volume of HBSS. A minimum of 6,000 cells per sample were analyzed. Each assay was performed at least three times, and the results are expressed as the mean ± standard deviation of the proportion of cells associated with bacteria.

Phagocytosis and killing by whole-blood leukocytes. The effects of complement deficiencies in mice deficient in C1qa, Bf, and Bf/C2 on the whole-blood killing capacity of *S. pneumoniae* were determined as described previously with minor modifications (6). Heparinized blood was collected by cardiac puncture. Blood killing assays were performed in polypropylene tubes containing 350 μl of mouse blood and 50 μl of *S. pneumoniae* to yield a final bacterial concentration of 10^4 CFU/ml. The samples were rotated end over end at 37°C, and aliquots were removed for viable plate count after 0, 60, and 120 min. Blood samples were tested on three occasions for 15 wild-type mice and 15 of each complement-deficient mice. The data are expressed as the mean ± standard deviation of the percentage of survival of the initial inoculum.

Quantitation of cytokine proteins in the middle ear lavage samples by ELISA. The middle ear lavage fluid samples from mice treated with *S. pneumoniae* type 6A were centrifuged at 500 × *g*, and the supernatants were aliquoted and frozen at −70°C. The concentrations of interleukin 1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), and monocyte chemotactic peptide 1 (MCP-1) in middle

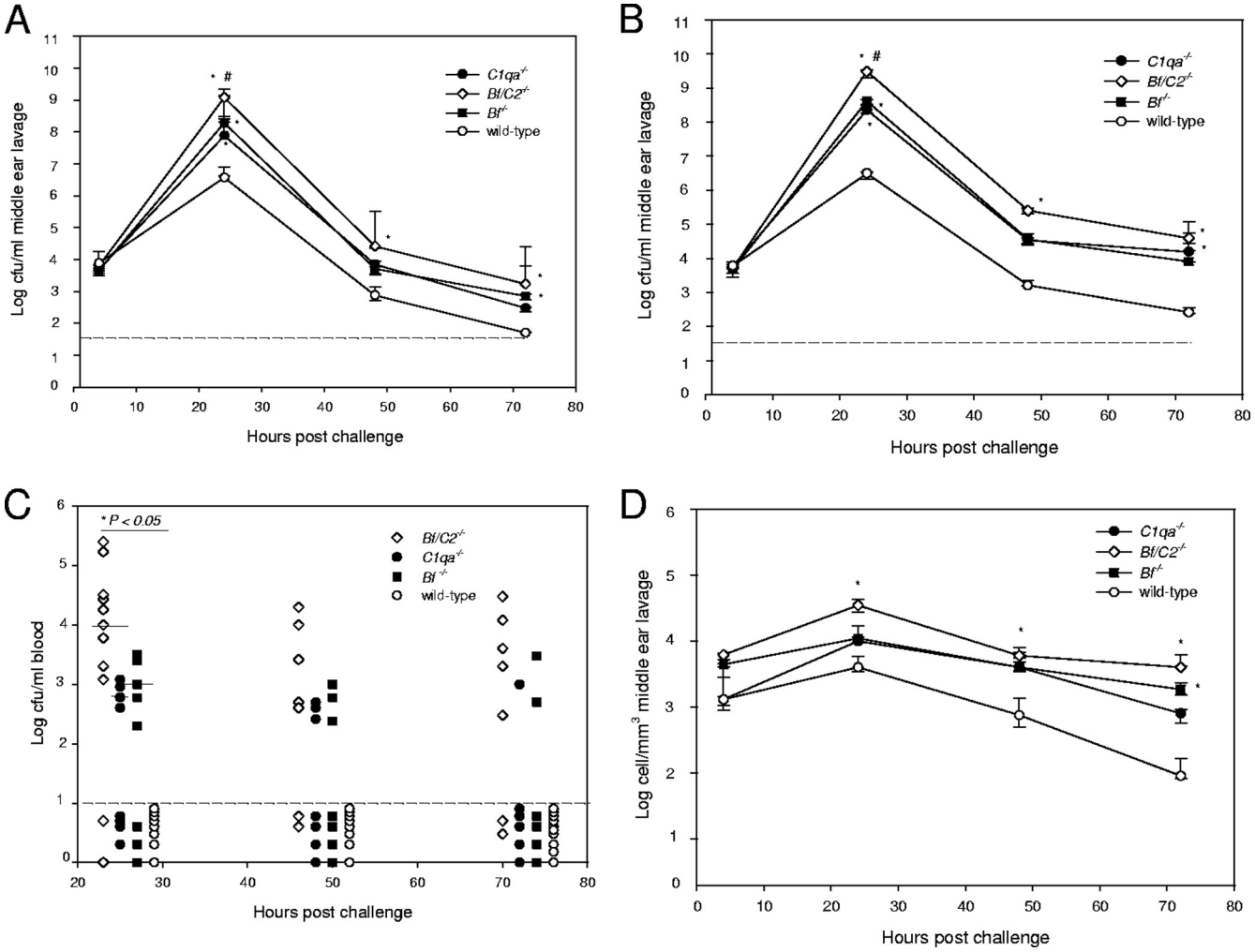

FIG. 1. (A and B) Survival of *S. pneumoniae* type 6A (A) and type 14 (B) in the middle ears of mice following transtympanical inoculation with approximately 1×10^3 CFU of *S. pneumoniae* bacteria. Each data point represents the mean number of CFU of *S. pneumoniae* (± SEM [error bar]) per milliliter of the middle ear lavage fluid samples from a total of 6 to 11 animals combined from two separate experiments. Values that are significantly different are indicated as follows: ∗, $P < 0.05$ for the comparison with the wild-type mouse cohort; #, $P < 0.05$ for the comparison of *Bf/C2*$^{-/-}$ mice with *C1qa*$^{-/-}$ or *Bf*$^{-/-}$ mice. The dashed line represents the detection limit of the assay. (C) Bacterial dissemination in blood. Blood samples were collected at 24, 28, and 72 h after transtympanical challenge with *S. pneumoniae* type 6A and cultured on Columbia CNA agar plates. The horizontal bar shows the mean numbers of bacterial titers after 24 h postinfection. Values that are significantly different are indicated as follows: ∗, $P < 0.05$ for the comparison of *Bf/C2*$^{-/-}$ mice with *C1qa*$^{-/-}$ or *Bf*$^{-/-}$ mouse cohort. The dashed line represents the detection limit of the assay. The values for mice with negative blood culture results are shown below the dashed line. (D) Accumulation of inflammatory cells in the middle ears of mice following transtympanical inoculation of *S. pneumoniae* type 6A. Each data point represents the mean concentration of inflammatory cells (± SEM) per cubic millimeter of the middle ear lavage fluid. These results were obtained from a total of 6 to 11 animals combined from two separate experiments. Values that are significantly different ($P < 0.05$) from the values for the wild-type mice are indicated (∗).

ear lavage fluid samples were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Statistical analysis. Data are presented as the mean ± standard error of the mean (SEM) or standard deviation of the mean (SD) as indicated. Data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) and the Holm-Sidak or Dunn's methods were used for the statistical analysis and pair-wise multiple comparisons. In all cases, a *P* value of <0.05 was set as the measure of statistical significance.

RESULTS

Deficiencies in C1q, Bf, and Bf/C2 impair clearance of *S. pneumoniae* from the middle ear and blood. To distinguish the ability of specific complement pathways to protect against AOM, we measured the clearance kinetics of *S. pneumoniae* type 6A and type 14 from the middle ear and blood for up to 72 h postinoculation (Fig. 1A and B). At 4 h postchallenge, concentrations of both strains in the middle ear lavage samples of *Bf/C2*$^{-/-}$ mice were 2-fold higher than those in mice in the other cohorts, but the differences were not statistically significant. None of the animals were bacteremic at this time. At 24 h postchallenge, the bacterial concentrations of both strains in the middle ear lavage samples reached a peak and fell thereafter. The highest concentrations of bacteria at 24 h were noted for *Bf/C2*$^{-/-}$ mice, followed by *Bf*$^{-/-}$ mice and *C1qa*$^{-/-}$ mice ($P < 0.05$ for all compared to wild-type mice). At 48 h postchallenge, the titers remained significantly higher for *Bf/*

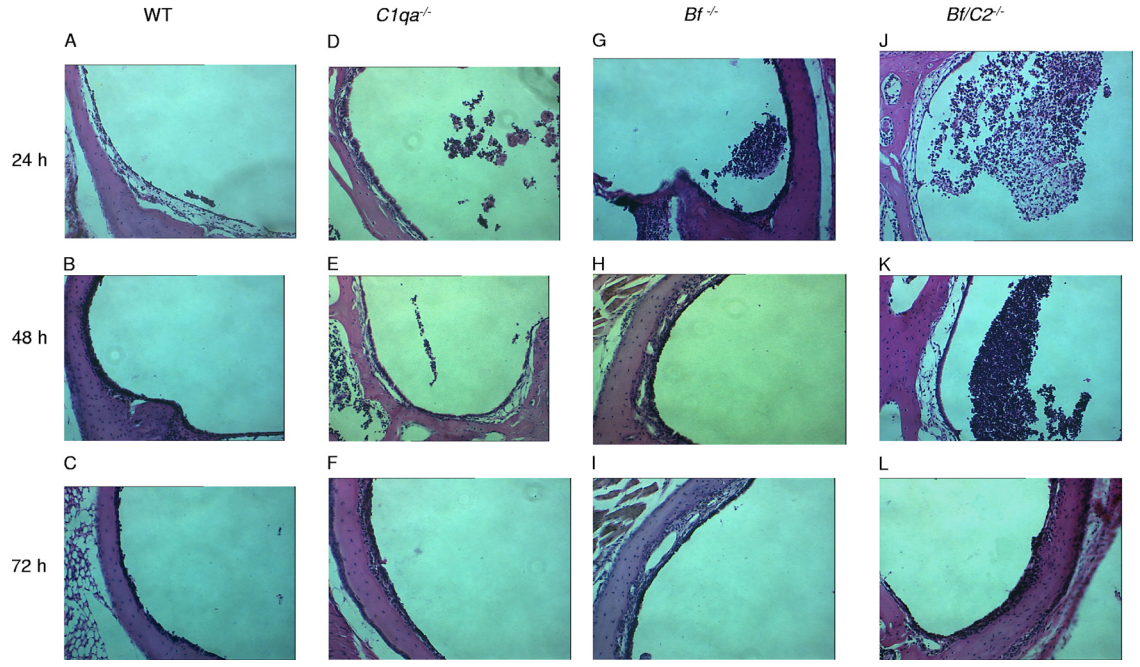

FIG. 2. Representative H&E-stained middle ear sections of the mice at 24, 48, and 72 h after transtympanical inoculation with *S. pneumoniae* 6A in the following cohorts of mice: wild-type (WT) (A to C), *C1qa*$^{-/-}$ (D to F), *Bf*$^{-/-}$ (G to I), and *Bf*/*C2*$^{-/-}$ (J to L). The inflammatory infiltrates in the middle ear epithelium were evident in the *Bf*/*C2*$^{-/-}$ mice. Magnification, ×200.

C2$^{-/-}$ mice than for wild-type mice ($P < 0.05$). At 72 h postchallenge, the titers of type 6A in *Bf*/*C2*$^{-/-}$and *Bf*$^{-/-}$ mice were significantly higher than those in wild-type mice ($P < 0.05$ for all compared to wild-type mice). There were no significant differences between *C1qa*$^{-/-}$ and *Bf*$^{-/-}$ mice at the time points examined. *S. pneumoniae* type 6A cells were eliminated from the middle ear in nine out of 10 wild-type mice at 72 h postchallenge, but in none of the complement-deficient mice. Though *S. pneumoniae* type 14 remained at a low count in all wild-type mice at 72 h postinoculation, the bacterial titers for *Bf*/*C2*$^{-/-}$and *C1qa*$^{-/-}$ mice were significantly higher than those for wild-type mice ($P < 0.05$ for all compared to wild-type mice).

Blood culture titers of strain type 6A for each cohort are shown in Fig. 1C. Persistent bacteremia was noted for all the cohorts of the complement-deficient mice across the 24-, 48-, and 72-h time periods. *S. pneumoniae* type 6A was isolated from the blood samples of 76% of *Bf*/*C2*$^{-/-}$ mice, 38% of *C1qa*$^{-/-}$ mice, 43% of *Bf*$^{-/-}$ mice, and 0% of wild-type mice during the 72-h observation period ($P < 0.001$ for complement-deficient versus wild-type mice). The frequency of positive blood cultures was not significantly different in *Bf*$^{-/-}$ and *C1qa*$^{-/-}$ mice. *Bf*/*C2*$^{-/-}$ mice had significantly higher blood titers at 24 h postchallenge than either *C1qa*$^{-/-}$ or *Bf*$^{-/-}$ mice ($P < 0.05$). A similar pattern was observed in the cohorts of mice infected with *S. pneumoniae* type 14; bacteria were isolated from the blood samples from 35% of *Bf*/*C2*$^{-/-}$ mice, 18% of *C1qa*$^{-/-}$ mice, 20% of *Bf*$^{-/-}$ mice, and 0% of wild-type mice during the 72-h observation period.

Effect of complement deficiencies on the inflammatory cellular response in the middle ear. Cell counts were performed on the middle ear lavage samples to compare the effects of the various complement deficiencies in C1qa, Bf, and Bf/C2 on recruitment of inflammatory cells in the middle ear after inoculation with *S. pneumoniae* type 6A (Fig. 1D). The cell counts reached a peak at 24 h and fell thereafter. There were no significant differences in the magnitude of the inflammatory cells at 4 h postchallenge. At 24, 48, and 72 h postchallenge, there were significantly more inflammatory cells in the lavage samples obtained from *Bf*/*C2*$^{-/-}$ mice than from wild-type mice ($P < 0.05$). There were significantly more inflammatory cells in the lavage samples collected from *Bf*$^{-/-}$ mice than wild-type mice at 72 h postchallenge ($P < 0.05$). The same trend was noted in the cohorts of mice infected with *S. pneumoniae* type 14 (data not shown). These data indicate that deficiencies in the classical and alternative complement pathways tended to enhance rather than impair inflammatory cell recruitment.

Histopathological evaluation. Inflammatory and structural changes in the middle ears of complement-deficient and wild-type mice infected with type 6A were assessed at 24, 48, and 72 h postinfection (Fig. 2). The middle ear histology was found to be normal in all mice except for minimal cellular inflammatory infiltrates within the epithelium or subepithelium at 4 h postinoculation (data not shown). At 24 h postinoculation, more inflammatory cells and mucosal thickening were noted in the middle ear spaces of mice of the complement-deficient cohorts than in wild-type mice. The changes in the middle ear mucosa were much more pronounced and persistent in *Bf*/*C2*$^{-/-}$ mice than in wild-type mice (Fig. 2J and K). The magnitude of the influx of cells observed on histological examination correlated with the inflammatory cell counts in the middle ear lavage samples as described above.

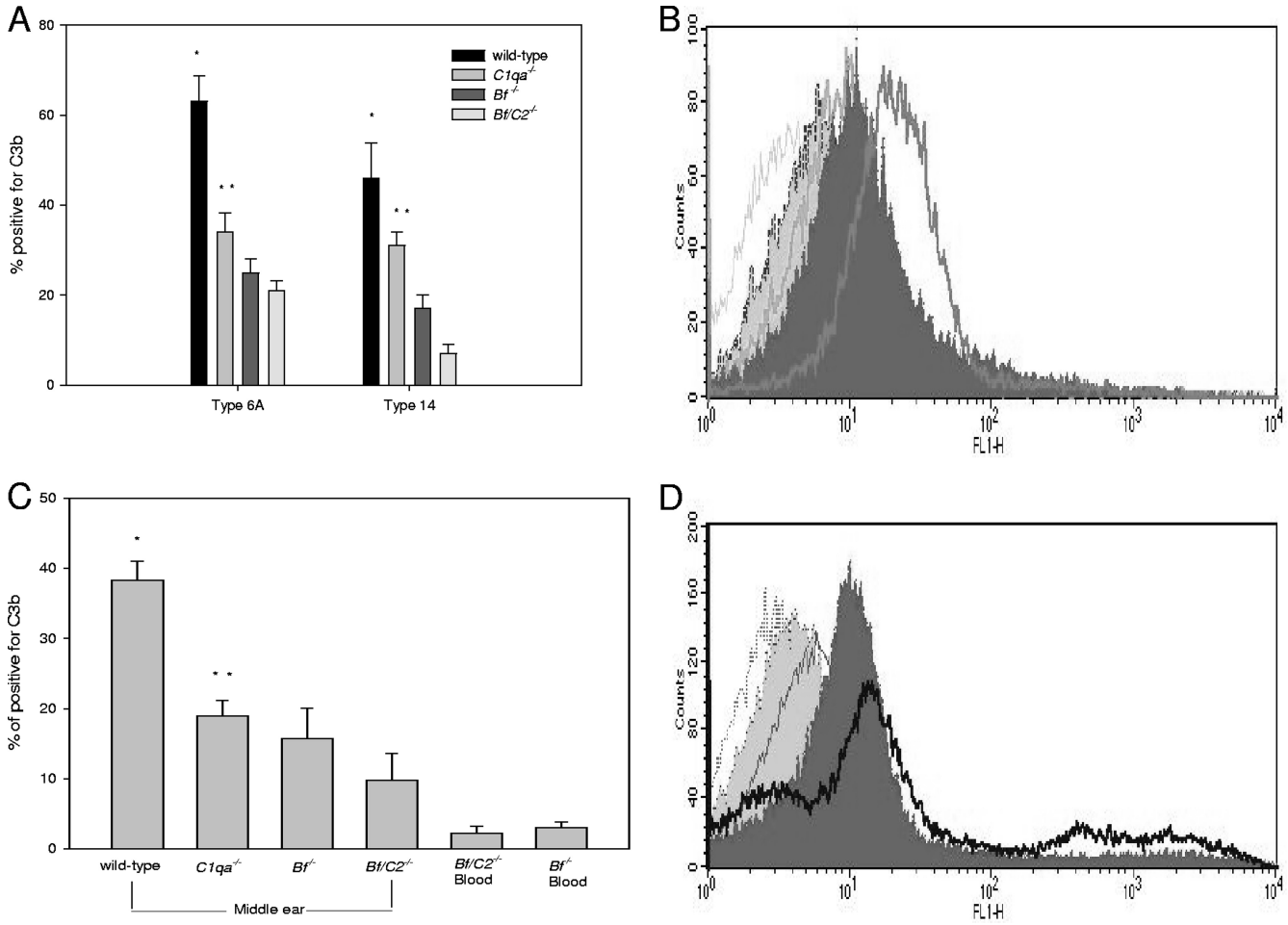

FIG. 3. C3b deposition on *S. pneumoniae*. The freshly grown bacterial cells were opsonized with wild-type or complement-deficient mouse sera. C3b fragments on the bacterial surfaces were detected in a flow cytometric assay with anti-mouse C3 antibody. (A) Proportion of *S. pneumoniae* positive for C3b. The results collected from three different experiments are expressed as means plus standard deviations (error bars). Values that are significantly different are indicated as follows: *, $P < 0.001$ compared with values determined for the complement-deficient groups; **, $P < 0.05$ compared with values for the $Bf/C2^{-/-}$ group. (B) A representative histogram of C3 deposition on *S. pneumoniae* type 6A fluorescence indicates, in order from left to right, the peak values for no serum, $Bf/C2^{-/-}$ mice, $Bf^{-/-}$ mice, $C1qa^{-/-}$ mice, and wild-type mice. (C) C3b deposition on *S. pneumoniae* type 6A recovered from the middle ear lavage fluid and blood samples from wild-type and complement-deficient mice at 24 h postchallenge. The results collected from three different experiments are expressed as means plus standard deviations (error bars). Values that are significantly different are indicated as follows: *, $P < 0.001$ compared with values determined for the complement-deficient groups. **, $P < 0.05$ compared with values for the $Bf/C2^{-/-}$ group. (D) Representative histogram of C3 deposition on the strain type 6A *in vivo*. Fluorescence indicates, in order from left to right, the peak values for the blank, $Bf/C2^{-/-}$ mice, $Bf^{-/-}$ mice, $C1qa^{-/-}$ mice, and wild-type mice.

Effects of deficiencies in complement pathways on C3b deposition on *S. pneumoniae* and phagocytosis. Deposition of C3b on culture-grown *S. pneumoniae* by sera obtained from the complement-deficient and wild-type mice is shown in Fig. 3A and B. The proportions of *S. pneumoniae* type 6A that fixed C3b on their surfaces were 63% for sera from wild-type mice, 34% for sera from $C1qa^{-/-}$ mice, 26% for sera from $Bf^{-/-}$ mice, and 21% for sera from $Bf/C2^{-/-}$ mice ($P < 0.001$ for complement-deficient versus wild-type mice in all cases). The proportions of strain type 14 that fixed C3b on their surfaces were 45% for sera from wild-type mice, 31% for sera from $C1qa^{-/-}$ mice, 17% for sera from $Bf^{-/-}$ mice, and 5% for sera from $Bf/C2^{-/-}$ mice ($P < 0.001$ for complement-deficient versus wild-type mice in all cases).

Deposition of C3b on *S. pneumoniae* type 6A recovered from the middle ear lavage and blood samples at 24 h postchallenge obtained from the complement-deficient and wild-type mice is shown in Fig. 3C and D. *S. pneumoniae* recovered from the middle ear bound C3b in the same sequential order as the culture-grown bacteria. Only a small percentage of *S. pneumoniae* recovered from the blood samples of $Bf/C2^{-/-}$ and $Bf^{-/-}$ mice bound C3b. These findings indicate that the intact complement pathway accounted for far more deposition of C3b on *S. pneumoniae* than either the classical or alternative pathway. They also suggest that pneumococci isolated from blood from $Bf/C2^{-/-}$ and $Bf^{-/-}$ mice may have lost the ability to bind C3b.

Opsonophagocytosis by sera from $C1qa^{-/-}$ mice, $Bf^{-/-}$ mice, $Bf/C2^{-/-}$ mice, and wild-type mice was determined using HL-60 cells, a human tissue culture cell line (Fig. 4A and B).

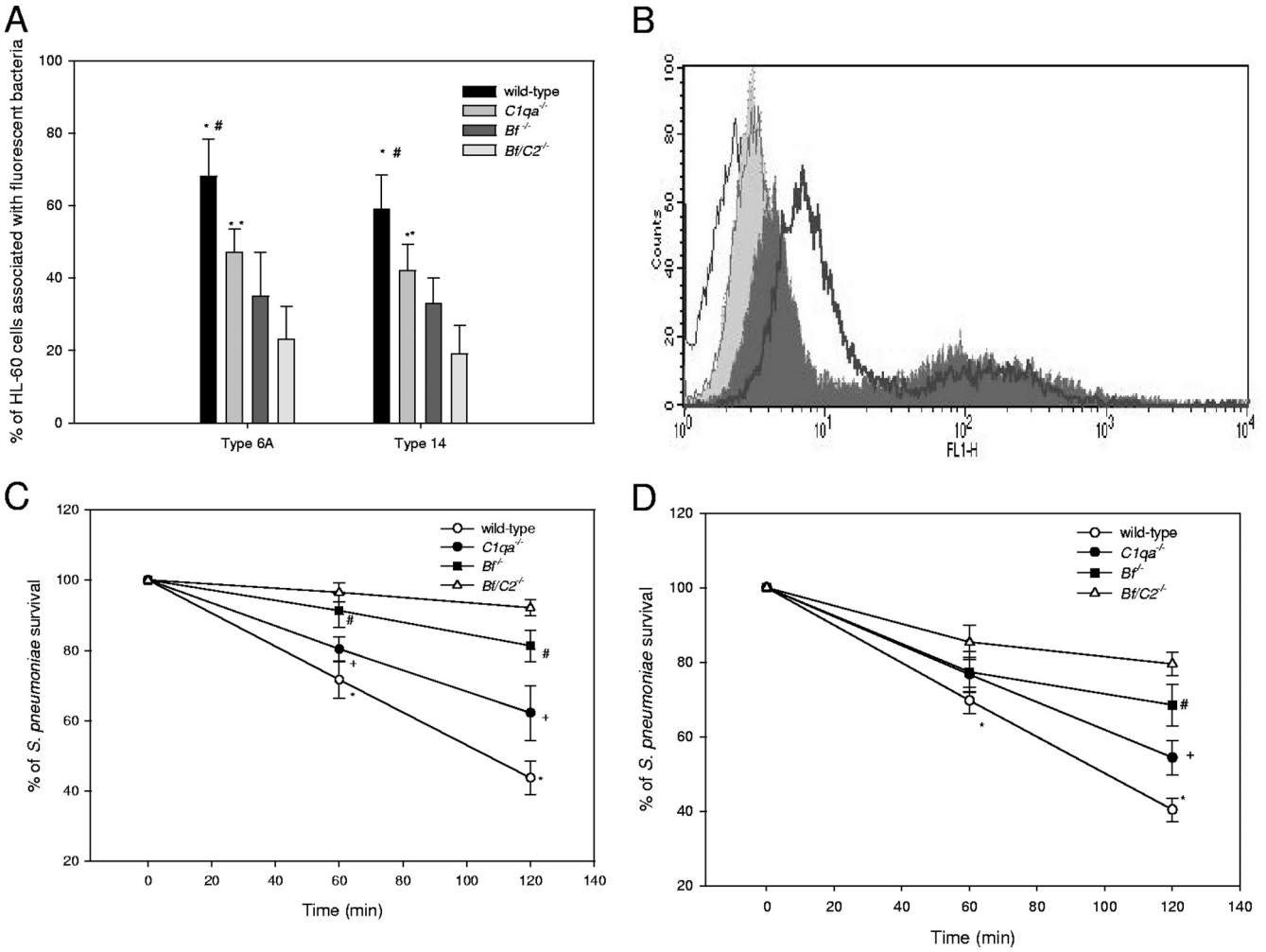

FIG. 4. Effects of complement deficiencies on opsonophagocytosis and whole-blood killing of *S. pneumoniae*. (A) Proportion of HL60 cells associated with fluorescent *S. pneumoniae* after incubation in sera from wild-type and complement-deficient mice. The results collected from three different experiments are expressed as means plus standard deviations (error bars). Values that are significantly different are indicated as follows: *, $P < 0.001$ compared with values determined for the complement deficiencies in Bf/C2 and Bf groups; #, $P < 0.05$ compared with values for the $C1qa^{-/-}$ group; **, $P < 0.05$ compared with values for the $Bf/C2^{-/-}$ group. (B) A representative histogram of fluorescence indicates, in order from left to right, the peak values for the blank, $Bf/C2^{-/-}$ mice, $Bf^{-/-}$ mice, $C1qa^{-/-}$ mice, and wild-type mice. (C and D) Killing of *S. pneumoniae* type 6A (C) and type 14 (D) with whole blood collected from wild-type and complement-deficient C1qa, Bf, and Bf/C2 mice. The data collected from three different experiments are expressed as means ± standard deviations (error bars) of the percent survival calculated as mean number of CFU after 60 and 120 min divided by the number of CFU in the inoculum at start × 100. Values that are significantly different are indicated as follows: *, $P < 0.001$ for the comparison of wild-type versus $Bf/C2^{-/-}$, $Bf^{-/-}$, and $C1qa^{-/-}$ cohorts; +, $P < 0.01$ for the comparison of $C1qa^{-/-}$ versus $Bf/C2^{-/-}$ or $Bf^{-/-}$ cohort; #, $P < 0.05$ for the comparison of $Bf^{-/-}$ versus $Bf/C2^{-/-}$ cohort.

The percentages of uptake of type 6A and type 14 by phagocytic cells were 68 and 59 for wild-type mouse sera, 47 and 42 for $C1qa^{-/-}$ mouse sera, 35 and 33 for $Bf^{-/-}$ mouse sera, and 26% and 19% for $Bf/C2^{-/-}$ mouse sera, respectively. The differences in uptake between wild-type mouse sera and sera from complement-deficient mice were statistically significant: wild-type versus $Bf^{-/-}$ and $Bf/C2^{-/-}$ mice, $P < 0.001$; and wild-type versus $C1qa^{-/-}$ mice, $P < 0.05$. These data indicate that the reduced capacity of C3b deposition on *S. pneumoniae* by $Bf/C2^{-/-}$, $Bf^{-/-}$, and $C1qa^{-/-}$ mouse serum accounted for decreased phagocytosis.

The capacity of whole blood obtained from wild-type and complement-deficient mice to kill *S. pneumoniae* is shown in Fig. 4C and D. Blood from wild-type mice killed 56% and 59% of the inocula of *S. pneumoniae* type 6A and type 14, respectively, after 2 h of incubation at 37°C. In contrast, blood from $C1qa^{-/-}$, $Bf^{-/-}$, and $Bf/C2^{-/-}$ animals killed only 38% and 47%, 19% and 39%, and 8% and 19% of the *S. pneumoniae* type 6A and type 14 cells, respectively ($P < 0.001$ in all cases compared with wild-type mice). The decreased bactericidal capacity of whole blood obtained from complement-deficient C1qa, Bf, and Bf/C2 mice is in accord with the results of opsonophagocytosis studies.

Cytokine production in the mouse middle ear. To determine whether enhanced cytokine production might explain the excessive inflammatory response in the middle ears of infected complement-deficient mice, we measured IL-6, IL-1β, TNF-α, and chemokine MCP-1 levels in the mouse middle ear lavage

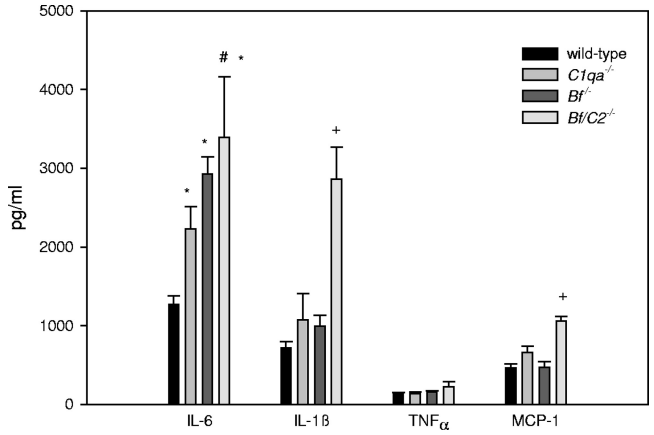

FIG. 5. Concentrations of cytokines in the middle ear lavage samples at 24 h postinfection with *S. pneumoniae* type 6A. Results are the mean concentrations of IL-1β, IL-6, and TNF-α (plus SEM [error bar]) in the middle ear lavage samples from two duplicate wells from two separate experiments. Values that are significantly different are indicated as follows: *, $P < 0.001$ compared to the values for the wild-type group; #, $P < 0.05$ compared to the values for the $C1qa^{-/-}$ group; +, $P < 0.001$ compared to the values for the wild-type, $Bf^{-/-}$, and $C1qa^{-/-}$ cohorts.

samples obtained at 24 h postinoculation from mice infected with strain type 6A (Fig. 5). IL-1β and MCP-1 levels were significantly higher in $Bf/C2^{-/-}$ mice than in wild-type mice, $C1qa^{-/-}$ mice, and $Bf^{-/-}$ mice ($P < 0.001$ in all cases). IL-6 levels were significantly higher in $Bf/C2^{-/-}$ mice than in wild-type and $C1qa^{-/-}$ mice ($P < 0.001$). Similarly, IL-6 levels were significantly higher in $Bf^{-/-}$ mice and $C1qa^{-/-}$ mice than in wild-type mice ($P < 0.001$ and $P < 0.05$, respectively). There were no significant differences in the low levels of TNF-α among wild-type and complement-deficient mice. The data are consistent with the finding of greater influx of inflammatory cells in the middle ears of complement-deficient mice compared to wild-type mice.

DISCUSSION

There is considerable evidence from clinical and experimental studies indicating that the complement system plays an important role in host defense against pneumococcal pneumonia and septicemia and that the classical pathway is the most important mode for activation of complement (4, 27). In the current study, we were able to show that C1qa protects mice from pneumococcal AOM. These observations are similar to those of other investigations in mouse models of pneumococcal pneumonia and septicemia (4). One of the most striking findings in the current study was that not only were *S. pneumoniae* cells able to survive longer in the middle ears of $Bf/C2^{-/-}$ mice and other complement-deficient mice than in the wild-type mice but they could also produce persistent bacteremia in complement-deficient mice. We were also able to demonstrate, using $Bf^{-/-}$ mice, that factor B may play a protective role at least equal to C1qa in the middle ear defense against *S. pneumoniae*. The alternative pathway has a dual functional role in pathogen recognition and amplification of complement activation initiated by the classical and lectin pathways (26). It is possible that the relatively different impact of factor B on immune defense in the mouse models of pneumococcal AOM and pneumonia can be attributed to anatomical differences and the site of inoculation. Unlike the lung, the middle ear contains few mucous glands, a low density of goblet cells, no organized lymphoid follicles, and few lymphocytes (14). Despite these anatomical differences, we found that the middle ear is able to mount an innate immune response to *S. pneumoniae* by activating the classical and alternative pathways. Our findings are consistent with other studies that have shown an increased susceptibility of $Bf/C2^{-/-}$ mice to *Candida albicans* compared to $C1qa^{-/-}$ mice (7). The finding that $Bf/C2^{-/-}$ deficiency significantly increases susceptibility to pneumococcal AOM more than $C1qa^{-/-}$ or $Bf^{-/-}$ deficiency indicates that the classical and alternative pathways are additive in clearing *S. pneumoniae* from the middle ear. C2 is involved in the common pathway for classical and lectin pathways. Previous reports have shown that mannan-binding lectin plays a minor role in the opsonophagocytosis of *S. pneumoniae* (3) and in host defense in the mouse model of pneumococcal pneumonia and septicemia (4). A recent report (20), however, suggests that the lectin pathway can function in the absence of C2 and/or C4 if the alternative pathway is intact. The current study was not designed to resolve the issue of whether the lectin pathway plays a part in the innate immune response during pneumococcal AOM. Further studies are needed using mannose-binding lectin-deficient mice to resolve this issue.

The differences in survival of *S. pneumoniae* in the middle ears of complement-deficient and wild-type mice prompted us to examine the mechanisms responsible for this phenomenon. Previous studies indicated that both bacterial and host factors could influence the *in vivo* survival of *S. pneumoniae*. Surface-exposed proteins, such as CbpA, PspA, and RlrA, have been shown to be complement resistance factors. A recent report (5) suggests that OM-specific genes of *S. pneumoniae* may also be required for pneumococcal replication and survival in the middle ear. In the current study, we compared the recruitment of inflammatory cells in the middle ears of complement-deficient and wild-type mice during the early stages of AOM. In contrast to a previous report that showed reduced recruitment of leukocytes into cerebrospinal fluid (CSF) in C3-deficient mice in a mouse model of pneumococcal meningitis (17), we found higher inflammatory cell concentrations in the middle ears of complement-deficient mice than in wild-type mice. These findings indicate that the recruitment of inflammatory cells is not impaired in complement-deficient mice. Furthermore, complement deficiencies in C1qa, Bf, and Bf/C2 had no impact on the impairment of cytokine production in the middle ear during the disease course. These findings are in accord with a previous report that demonstrated C3 was not required for neutrophil recruitment and cytokine (IL-6 and TNF-α) production in a mouse model of pneumococcal pneumonia (9). It appears that the influx of inflammatory cells may be driven by increased levels of cytokines and bacterial replication in the middle ear.

$Bf/C2^{-/-}$ mice were unable to clear bacteria from the middle ear and developed persistent bacteremia despite a robust recruitment of inflammatory cells into the infected middle ears in response to pneumococcal infection. This can be explained by the decreased ability of the sera of these mice to bind C3b to *S. pneumoniae* obtained either from broth cultures or from the

middle ear lavage and blood samples. Their inability to mount an adequate opsonophagocytic response was paralleled with their reduced ability to kill *S. pneumoniae*. This notion is supported by Melin et al. who found a clear association between the total amount of C3b detected on the bacterial surfaces and the sensitivity of the same strains to opsonophagocytosis (13). This is also consistent with the well-established concept that opsonophagocytosis is the primary mechanism for clearance of pneumococci. It is conceivable that our data indicate that opsonophagocytosis mediated by intact complement pathway is a major protection mechanism for host against *S. pneumoniae* in the mouse model of AOM.

In conclusion, our data indicate that deficiencies in the classical and alternative pathways contribute to increased survival of *S. pneumoniae* in the middle ear and occurrence of persistent bacteremia. The responsible mechanisms appear to be the reduction of C3-mediated opsonization and phagocytic killing. Other complement-independent mechanisms might also play roles and warrant further investigation.

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