INFECTION AND IMMUNITY, July 2011, p. 2578–2585 Vol. 79, No. 7
0019-9567/11/$12.00 doi:10.1128/IAI.00168-11

Essential Role of Factor B of the Alternative Complement Pathway in Complement Activation and Opsonophagocytosis during Acute Pneumococcal Otitis Media in Mice▽

Qian Li,[1,4] Yong Xing Li,[1] Gregory L. Stahl,[2] Joshua M. Thurman,[3] Yujuan He,[1,5] and Hua Hua Tong[1]*

Department of Otolaryngology, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210[1]; Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115[2]; Department of Medicine, University of Colorado—Denver, Aurora, Colorado 80045[3]; Department of Infectious Diseases, FuDan University affiliated Hua Shan Hospital, Shanghai, China[4]; and Department of Clinical Laboratory Medicine, Chongqing Medical University, Chongqing, China[5]

Received 18 February 2011/Returned for modification 18 March 2011/Accepted 7 April 2011

We recently reported that the complement system plays a pivotal role in innate immune defense against *Streptococcus pneumoniae* during acute otitis media (OM) in mice. The current study was designed to determine which of the complement pathways are activated during acute pneumococcal OM and whether components of complement are expressed in the middle ear epithelium. Gene expression was determined by quantitative PCR, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence staining. We found that *S. pneumoniae* induced increased gene expression of factor B of the alternative complement pathway and C3 in mouse middle ear epithelium. Activation of factor B and C3 in the middle ear lavage fluids was significantly greater than in simultaneously obtained serum samples as determined by Western blotting. Using mice deficient in complement C1qa, factor B, and factor B/C2, we found that complement C3 activation and opsonophagocytosis of *S. pneumoniae* were greatly attenuated in factor B- and factor B/C2-deficient mice. These findings support the concept that local complement activation is an important host innate immune response and that activation of the alternative complement pathway represents one of the innate immune defense mechanisms against pneumococcal infection during the early stage of acute OM.

Otitis media (OM) is one of the most common diseases in childhood. The monetary and social costs of the morbidity of the disease and hearing loss are considerable (8). Following the introduction of seven-valent pneumococcal conjugate (PCV7) in the United States, non-PCV7 serotypes replaced PCV serotypes as the major bacterial pathogens of OM in children (6). Clinical and experimental studies have shown that the middle ear epithelium plays an important role in recognizing invading otopathogens and subsequently in initiating the immune response. Despite advances in understanding the interaction of host defense systems and otopathogens, the mechanisms by which the middle ear epithelium serves as a first line of the host innate immune defense is not yet completely understood.

The complement system provides the major innate immune defense mechanism against pneumococcal infections (5, 12, 23). It is activated in response to infection by the classical, alternative, and lectin pathways. The classical pathway is initiated by the binding of C1q to antigen-antibody immune complexes or C-reactive protein, serum amyloid P, or components of bacterial cell wall. The lectin pathway is initiated by the binding of mannose-binding lectin (MBL) to carbohydrate groups on the surfaces of some pathogens. Increased C4 deposition is indicative of complement activation by either the classical or lectin pathway (21). In contrast, the alternative pathway is auto-activated by binding of spontaneously hydrolyzed C3 with factor B. This allows cleavage of factor B by factor D into the fragments Ba and Bb, generating C3bBb, the alternative pathway C3 convertase. In addition, the alternative pathway amplifies complement activation initiated through the classical/lectin pathways. Thus, increased production of the Bb fragment can be regarded as a marker of alternative pathway activation (21).

Brown and colleagues (5) reported that the classical pathway was the most important mode of complement activation in a mouse model of *Streptococcus pneumoniae* pneumonia and systemic infection. We found that deficiency in the alternative pathway also contributed to increased survival of *S. pneumoniae* in the middle ear and persistent bacteremia in a mouse model of pneumococcal OM (27). However, since the alternative pathway can be initiated by the classical pathway, it is still uncertain which pathways are necessary for complement activation by *S. pneumoniae* in the middle ear. The current study was designed to determine which complement pathways are activated during acute pneumococcal OM (AOM) and whether complement components are expressed in the middle ear epithelium.

The liver is the major site of complement synthesis. Extrahepatic tissue-specific complement synthesis provides a more immediate local immune response. Human, mouse, and rat

* Corresponding author. Mailing address: The Ohio State University, Department of Otolaryngology-Head and Neck Surgery, 915 Olentangy River Road, Suite 4000, Columbus, OH 43212. Phone: (614) 293-8103. Fax: (614) 293-5506. E-mail: tong.1@osu.edu.
▽ Published ahead of print on 18 April 2011.

lung epithelial cells synthesize complement proteins (2, 22). This appears to occur in OM as well since the middle ear is the uppermost part of the respiratory tract mucosal epithelium. Studies of children with chronic OM with effusion (OME) have demonstrated strong staining for complement fragments iC3b/C3c and weaker labeling for C3d and C9 on the surface of the middle ear mucosa. These findings indicate local complement activation in the middle ear mucosa (18, 19). To extend these observations, we evaluated the expression of six complement genes in the mouse middle ear epithelium during experimental pneumococcal OM. We found that factor B was the most highly induced gene, followed by C3, among the six genes examined in this study. We also found that complement activation was greater in middle ear lavage fluids than in serum during the first 72 h of infection. Furthermore, using mice deficient in C1qa (and thus unable to activate complement through the classical pathway), factor B (and thus unable to activate complement through the alternative pathway), or factor B/C2, we were able to demonstrate that complement activation in the middle ear depends in large part on the alternative pathway. However, while we have identified the critical role of alternative pathway activation in promoting *S. pneumoniae* opsonophagocytosis by neutrophils, the reduced opsonophagocytosis of *S. pneumoniae* in *C1qa*$^{-/-}$ mice suggests that the classical pathway also plays a significant role in the host innate immune mechanism during the early stage of pneumococcal OM.

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, Göteborg, Sweden (1, 27). The growth conditions and inocula were prepared as previously described (27).

Mice. Eight- to 12-week-old male or female C57BL/6 mice were used in this study. C57BL/6 mice homozygous for combined gene deficiencies of factor B and C2 (*Bf*/*C2*$^{-/-}$), C1qa (*C1qa*$^{-/-}$), and factor B (*Bf* $^{-/-}$) were generated as previously described (3, 15, 26). *C1qa*$^{-/-}$, *Bf*/*C2*$^{-/-}$, and *Bf* $^{-/-}$ mice were backcrossed at least nine generations onto the C57BL/6 background. Age- and sex-matched C57BL/6 mice, used as controls, were obtained from Taconic Farms and the Jackson Laboratories. All study procedures were approved by The Institutional Animal Care and Use Committee at The Ohio State University.

Mouse model of AOM. Acute pneumococcal OM was induced by direct bilateral transtympanic inoculation of the middle ear, as previously described (14). Briefly, 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 the inoculation of 5 μl of a suspension containing approximately 1×10^3 CFU of *S. pneumoniae* in sterile pyrogen-free saline. A control cohort of five mice was sham inoculated with 5 μl of diluent alone; an additional five mice were used as normal controls without injection. Mice were anesthetized and then sacrificed at 4, 24, 48, and 72 h postinoculation. Blood samples pooled from five mice with the same genetic background were obtained by cardiac puncture. Single-use aliquots of the sera were stored at −70°C. The middle ear space was lavaged prior to *in situ* lysis of the epithelium. The middle ear space was rinsed four times with 5 μl of sterile pyrogen-free saline; the washings were aspirated and pooled. Middle ear lavage samples were centrifuged at 500 × *g*, and single-use aliquots of the lavage samples were stored at −70°C. Following lavage, the middle ear epithelium was harvested by *in situ* lysis with 5 μl of lysis buffer from an RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (13). This process was repeated three times, and the lysates were aspirated, pooled, and stored at −70°C. Total RNA from the lysates pooled from five mice at each time point was isolated by using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen) and stored at −80°C until analyzed by real-time PCR.

Quantitation of complement component transcripts from the middle ear epithelium by real-time PCR. Real-time PCR assays were performed to quantitate C1q, C3, C4, factor B, factor H, and factor I transcripts. Total RNA from the middle ear lysate sample pooled from five mice was reverse transcribed with random hexamers by using a Superscript preamplification system (Invitrogen, Carlsbad, CA). Real-time PCR primers were selected for each gene by using Primer Express software (version 2.0; Applied Biosystems, Foster City, CA). Primer sets for the following genes were synthesized by Invitrogen: C1q (sense, 5′-GCGACCGAACCAGGTCAT-3′; antisense, 5′-TCTCGTTCGCGTTGGTGAT-3′), C3 (sense, 5′-CGTGAACAGGAGGAACTTAAGG-3′; antisense, 5′-ATGCTGCAGAAGGCTGGATT-3′), C4 (sense, 5′-TCAGGGTGTCAACTTGCTCTTC-3′; antisense, 5′-TGATCGGTCTGCACAAAGATG-3′), factor B (sense, 5′-GCATGGTGTGGGAGCATAAA-3′; antisense, 5′-GGCTTGCCATGGTTGCTTA-3′), factor H (sense, 5′-TCTCAGGCTCGTGGTCAGAA-3′; antisense, 5′-CCAGGGCGGCATTTGTAG-3′), factor I (sense, 5′-ACTGCTGCGCACTGTGTCA-3′; antisense, 5′-AAAGCCGTCCAGACTTGGTAAC-3′), and β-actin (sense, 5′-ACTGGGACGACATGGAGAAGA-3′; antisense, 5′-GCCACACGCAGCTCATTGTA-3′). Reactions were performed in a 50-μl volume that included diluted cDNA sample, primers, and SYBR green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. Real-time PCR amplifications were performed on an Applied Biosystems Prism 7900 HT Sequence Detector according to the manufacturer's instructions. All data were normalized to the β-actin mRNA levels. Relative changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method (where C_T is threshold cycle) as previously described (28) and expressed as the *n*-fold difference relative to cDNA from normal control mice prepared in parallel with the experimental cDNAs.

Quantitation of C3 proteins in the middle ear lavage and serum samples by ELISA. Concentrations of C3 in the middle ear lavage samples pooled from five mice were measured by use of commercial enzyme-linked immunosorbent assay (ELISA) kits (GenWay Biotech, San Diego, CA), according to the manufacturer's instructions. Middle ear lavage samples pooled from five sham-inoculated animals served as the controls.

Quantitation of albumin level in the middle ear lavage and serum samples and determination of local production of C3 in the middle ear. Albumin levels in middle ear lavage fluid and serum samples (pooled from five mice in each group) at 0, 4, 24, 48, and 72 h postinoculation were analyzed using a mouse albumin ELISA kit (Immunology Consultants Laboratory, Newberg, OR).

To determine whether increased concentration of C3 in the middle ear lavage samples was due to transudation from serum during infection, middle ear lavage/serum albumin ratios were calculated and compared with middle ear lavage/serum C3 ratios to assess the contribution of transudation from serum and to estimate the degree of local production, as described previously (25). Proteins with a secretion/serum ratio less than that of albumin are said to be present by simple diffusion, whereas those whose ratio is greater than that of albumin are thought to be present partly as a result of local production (25).

Immunohistochemistry. Temporal bones from 3 mice in each cohort were removed immediately after sacrifice at 24 h postchallenge. The samples were processed as described previously with minor modifications (27). The middle ear sections were deparaffinized and rehydrated through Histoclear and a graded alcohol series. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), and the sections were incubated with 0.05% trypsin solution (Invitrogen) at 37°C for 20 min to unmask antigens. The sections were then blocked with PBS (pH 7.2) containing 1% bovine serum albumin (BSA), 5% donkey serum, and 0.3% Triton X-100 for 1 h. The sections were then incubated at 4°C overnight with primary antibodies: goat anti-human factor B polyclonal antibody (1:200; Quidel, San Diego, CA), goat anti-mouse C3 (1:200; MP Biomedicals, Solon, OH), or rat anti-mouse C4 (1: 50; Hycult Biotech, Uden, Netherlands). Samples were washed and incubated with 1:500 dilutions of secondary antibodies conjugated to DyLight 488 or DyLight 594 (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 h and then incubated with 4′,6′-diamidino-2-phenylindole ([DAPI] 1:10,000; Invitrogen) at room temperature for 2 min. The sections were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Immunostained samples were examined with a Zeiss Axioskop microscope equipped with an Olympus Magnafire low-light color digital camera or with an Olympus Flowview 1000 laser scanning confocal microscope.

Western blotting. To evaluate the kinetics of factor B protein production during OM, equal volumes (25 μl) of middle ear lavage fluid samples pooled from five mice at 0, 4, 24, 48, and 72 h were subjected to SDS-PAGE. For comparison of complement activation in the middle ears and serum samples, equal amounts of protein (30 μg) from mouse middle ear lavage fluid and serum samples pooled from 4 to 5 mice were loaded on an SDS-PAGE gel. Samples were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked and then incubated with goat anti-human factor B polyclonal antibody (1:1,000; Quidel) or goat anti-mouse C3 (1:1,000; MP Biomedicals) in TBST (Tris-buffered saline supplemented with Tween 20) containing 5% dry milk at 4°C overnight. Blots were then washed and incubated with horserad-

ish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:10,000; Calbiochem, San Diego, CA) diluted in TBST containing 2% dry milk. The membranes were developed with a chemiluminescent detection system (ECL; GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol.

Analysis of inflammatory cells in the middle ear lavage fluid. The inflammatory cells of middle ear lavage fluid samples were collected from five wild-type or complement-deficient mice at 24 h postinfection and pooled. The cells were fixed with 2% paraformaldehyde, then blocked with 2.4G2 monoclonal antibody (MAb) to mouse Fcγ receptor II/III (BD Biosciences, San Diego, CA), and stained with phycoerythrin (PE)-conjugated antibody to CD11b and with fluorescein isothiocyanate (FITC)-conjugated antibody to Ly6G (BD Biosciences). The stained cells were analyzed using a FACSCalibur.

***In vivo* phagocytosis of *S. pneumoniae* by neutrophils in the middle ear.** The inflammatory cells of the middle ear lavage fluid samples from five wild-type or complement-deficient mice were collected at 24 h postinfection and pooled. The inflammatory cells were fixed in 2% paraformaldehyde. The cytospin preparations were blocked with PBS with 3% BSA and then incubated with a rabbit polyclonal antibody to pneumococcal polysaccharide (Statens Serum Institute, Copenhagen, Denmark) and rat anti-mouse C3/iC3b/C3c (Cell Sciences, Canton, MA). Secondary antibodies were donkey anti-rabbit IgG coupled with DyLight 594 and donkey anti-rat IgG conjugated with DyLight 488 (Jackson ImmunoResearch Laboratories). The slides were washed, counterstained with DAPI (Invitrogen) for nuclei, and evaluated.

For a quantitative analysis of *S. pneumoniae* phagocytosis by neutrophils *in vivo*, the middle ear lavage samples from five wild-type and complement-deficient mice were pooled at 24 h postinfection. Red cells were lysed with red blood cell (RBC) lysis solution (Qiagen), and the inflammatory cells were washed, resuspended at a density of 1×10^6 cells in 100 μl of Dulbecco's PBS (DPBS) containing 0.2% BSA. The cells were then lysed with 0.1% Triton X-100. The total numbers of *S. pneumoniae* bacteria (attached to and internalized in the cells) were determined by plate count. To determine the number of *S. pneumoniae* cells phagocytosed by neutrophils, 1×10^6 cells were incubated with gentamicin (100 μg/ml) for 1 h at 37°C to kill extracellular *S. pneumoniae*. Cells were washed and then lysed with 0.1% Triton X-100. The number of phagocytosed *S. pneumoniae* cells was determined by plate count.

Statistical analysis. Data are presented as the mean ± the standard error of the mean (SEM) or standard deviation of the mean (SD) as indicated in the figure legends. Data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). A Student's *t* test was used for comparison of gene expression between experimental and control cohorts. One-way analysis of variance (ANOVA) and the Holm-Sidak or Dunn's methods were used for the statistical analysis of multiple cohort and pairwise comparisons. In all cases, a P value of <0.05 was set as the measure of significance.

RESULTS

***S. pneumoniae* induced an increase in gene and protein expression of factor B and C3 in the mouse middle ear epithelium following transtympanic inoculation.** Real-time PCR was used to examine the changes in gene expression for several complement components. Expression of classical pathway components (e.g., C1q and C4), the alternative pathway (factor B), C3, and the inhibitory regulators factor H and factor I in the middle ear epithelium of wild-type and complement-deficient mice was evaluated. *S. pneumoniae* induced a rapid upregulation of factor B mRNA at 4 h in the wild-type mice. Expression peaked at 24 h and declined thereafter. A modest increase in C3 gene expression was induced at 24 and 48 h postinfection in wild-type mice (Fig. 1A). Similar kinetics for factor B and C3 gene expression were seen in *C1qa*$^{-/-}$ mice (Fig. 1B). As expected, factor B gene expression was not detected in *Bf*$^{-/-}$ and *Bf*/*C2*$^{-/-}$ mice (data not shown). Modest upregulation of C3 mRNA was observed in *Bf*$^{-/-}$ mice at 24 and 48 h following inoculation of *S. pneumoniae* (3.0- and 3.6-fold increases, respectively; $P < 0.05$) but not in *Bf*/*C2*$^{-/-}$ mice (data not shown). There was no significant alteration in gene expression of C1q, C4, factor H, and factor I in middle ear epithelium in wild-type or complement-deficient mice during the 72-h observation period (data not shown).

The factor B protein levels in the middle ear lavage samples pooled from five wild-type mice were evaluated by Western blotting. Equal volumes of the middle ear lavage samples at different time points postinfection were subjected to SDS-PAGE. Factor B was immunodetected at 0 h, indicating a basal constitutive expression of this protein. A significant increase of factor B was evident during the course of OM (Fig. 1C). In addition, intensive immunofluorescence staining of factor B proteins was noted in the cytoplasm of the middle ear epithelium in wild-type and *C1qa*$^{-/-}$ mice (Fig. 1E). Factor B protein was not detected in the middle ear lavage and tissue samples from *Bf*$^{-/-}$ and *Bf*/*C2*$^{-/-}$ mice (data not shown). These results correlated with induction of factor B gene expression as detected by real-time PCR.

A significant increase in C3 concentration in the middle ear lavage samples in infected cohorts relative to sham-inoculated controls was evident ($P < 0.05$ in all cases) (Fig. 1D). In addition, at 24 and 48 h postinfection, C3 concentrations in the middle ear lavage samples from wild-type mice were significantly higher than in the complement-deficient mice ($P < 0.05$). In addition, the middle ear lavage fluid/serum ratios of C3 were greater than the ratios of albumin (Table 1). Our data suggest that local production of C3 may contribute in part to increased C3 concentration in the middle ear.

Alternative complement pathway activation in the middle ear lavage and serum samples during acute pneumococcal OM. To investigate whether the increased factor B gene expression by middle ear epithelium *in vivo* results in alternative complement pathway activation locally during OM, factor B cleavage fragments in the middle ear lavage and serum samples were analyzed by Western blotting. We found that factor B, Bb, and Ba fragments were prominent in the middle ear lavage samples from wild-type and *C1qa*$^{-/-}$ mice (Fig. 2). Factor B, Bb, and Ba were not detected by Western blotting in *Bf*$^{-/-}$ and *Bf*/*C2*$^{-/-}$ mice (data not shown). In addition, most serum factor B was in the uncleaved form, suggesting that alternative pathway activation in the middle ear is a localized phenomenon.

Factor B deficiency decreases C3 activation during acute pneumococcal OM. The complement system activation in response to invading pathogens is mediated through the classical, alternative, and lectin pathways. Each pathway converges at the C3 convertase level, resulting in C3 cleavage. To further assess the specific complement pathway activation involved in OM, levels of C3 breakdown products in middle ear lavage samples and those in simultaneously collected blood serum samples were evaluated by Western blotting. The wild-type mice showed increased levels of C3 cleavage fragments as early as 4 h postinfection in the middle ear lavage and serum samples at 24, 48, and 72 h postinfection (Fig. 3A). The levels of C3 cleavage fragments in the middle ear lavage samples were much higher than in the serum samples (Fig. 3A). *C1qa*$^{-/-}$ mice showed a similarly intense level of C3 cleavage fragments in the middle ear lavage sample compared with that in the wild-type mice (Fig. 3B). This finding indicates a lesser impact of the classical pathway on complement C3 activation. In contrast, lower levels of C3 cleavage fragments in the middle ear lavage samples of *Bf*$^{-/-}$ mice were detected than in samples of wild-type and *C1qa*$^{-/-}$ mice (Fig. 3C). *Bf*/*C2*$^{-/-}$ mice, which lack

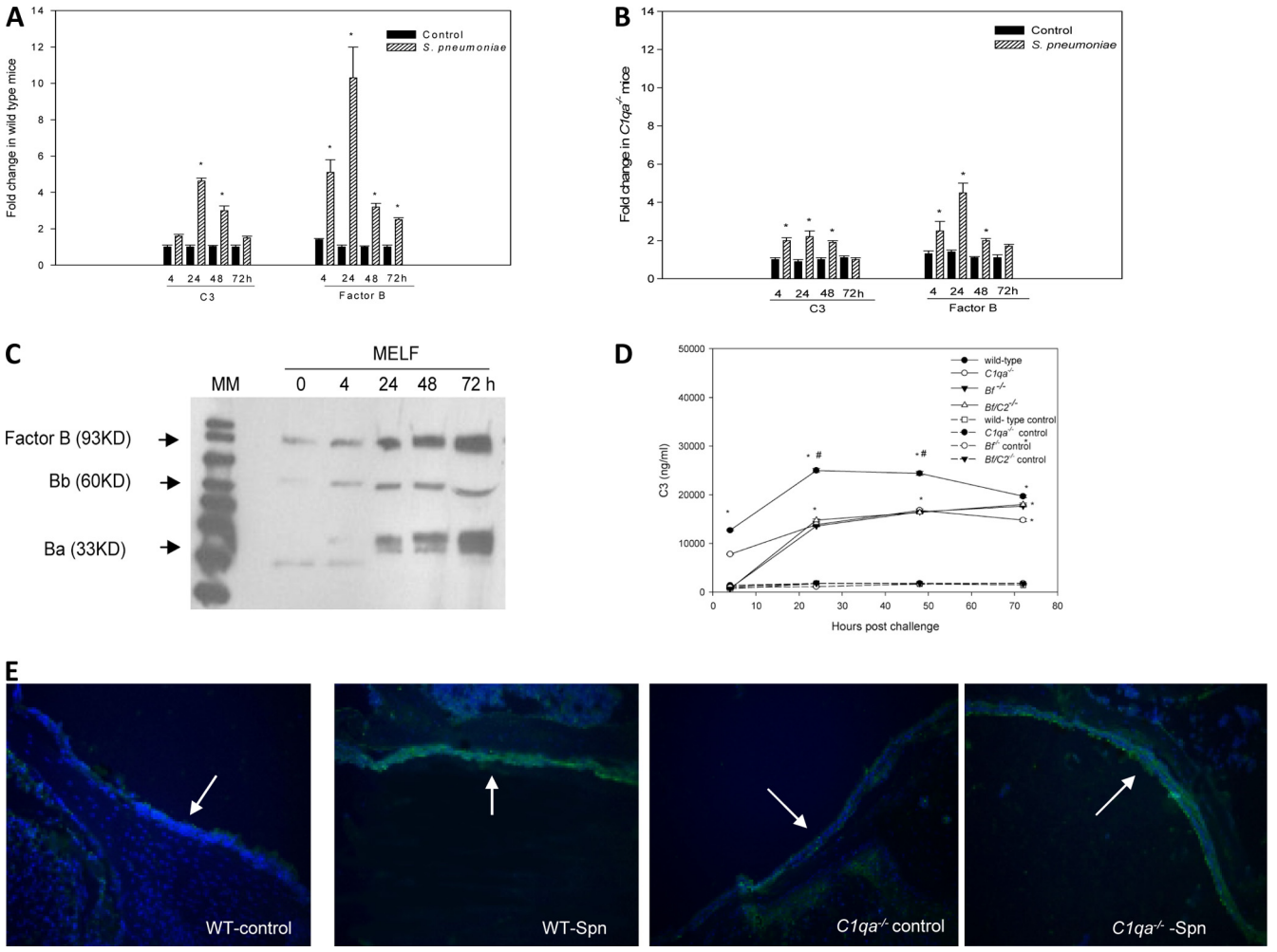

FIG. 1. Gene and protein expression of complement factor B and C3. Induction of gene expression as measured by real-time PCR on total RNA samples by direct *in situ* lysis of middle ear mucosa subsequent to lavage at 4, 24, 48, and 72 h following transtympanic inoculation with *S. pneumoniae* type 6A bacteria in wild-type (A) and *C1qa*$^{-/-}$ mice (B). Results are the mean fold changes in C3 and factor B transcript levels (± SEM) from duplicate samples pooled from five mice from two separate experiments. ∗, $P < 0.05$ compared with the values determined for the sham control cohorts at each time point. (C) Levels of factor B protein in mouse middle ear lavage fluid (MELF) samples pooled from five wild-type mice at 0, 4, 24, 48, and 72 h after transtympanic challenge with *S. pneumoniae* type 6A detected by Western blotting. Similar data were obtained in two independent experiments. MM, molecular mass. (D) Concentrations of C3 in the middle ear lavage fluid samples. Results are the mean concentration of C3 (± SEM) in the middle ear lavage samples pooled from five mice from two separate experiments. ∗, $P < 0.05$ for the comparison with each sham control cohort; #, $P < 0.05$ for the comparison of wild-type mice with *C1qa*$^{-/-}$, *Bf*$^{-/-}$, and *Bf*/*C2*$^{-/-}$ mice. (E) Increased factor B immunofluorescence staining in the middle ear epithelium in wild-type and *C1qa*$^{-/-}$ mice at 24 after transtympanic inoculation with *S. pneumoniae* type 6A compared with the sham control cohorts. Arrows indicate the middle ear mucosa (magnification, ×200). Representative images from three mice are shown.

both the classical and alternative pathways, were found to have an uncleaved form of C3 in both samples, in contrast to wild-type mice (Fig. 3D).

Additional evidence for complement activation in wild-type and complement-deficient mice was obtained by dual immunofluorescence staining for C4 and C3 deposition on middle ear epithelium at 24 h postinfection (Fig. 3E). The sham-inoculated wild-type mice showed faint C3 and C4 deposition

TABLE 1. Middle ear lavage fluid/serum ratios of C3 corrected for the ratios of albumin in wild-type mice

Parameter[a]	Value at the indicated time (h) postinfection[b]				
	0	4	24	48	72
C3 ratio (MELF/serum)	0.36 (0.002)	0.42 (0.0034)	0.79 (0.006)	0.55 (0.007)	0.98 (0.002)
Albumin ratio (MELF/serum)	0.20 (0.001)	0.32 (0.004)	0.44 (0.001)	0.50 (0.002)	0.68 (0.001)
C3 ratio corrected for albumin[c]	1.92 (0.01)	1.54 (0.02)	1.85 (0.08)	1.18 (0.07)	1.48 (0.05)

[a] MELF, middle ear lavage fluid.
[b] Samples were pooled from five mice from two independent studies. Values are means (SEM).
[c] The C3 ratio (MELF/serum) was divided by the albumin ratio (MELF/serum) to obtain the C3 ratio corrected for albumin.

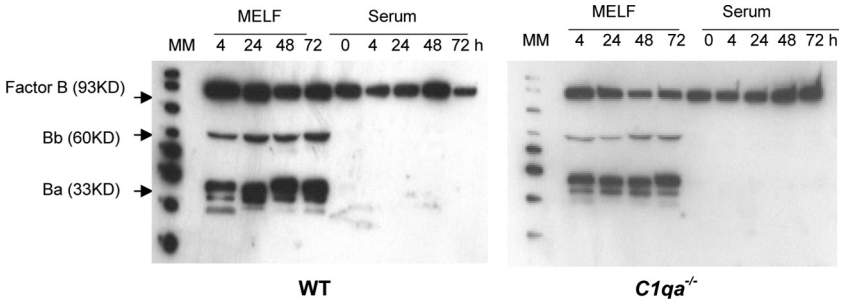

FIG. 2. Western blot analysis of factor B activation. Approximately 30 μg of protein from the middle ear lavage fluid (MELF) samples and corresponding serum samples collected and pooled from five mice at 4, 24, 48, and 72 h postinfection was subjected to SDS-PAGE. Bb and Ba were detected in the MELF samples in wild-type (WT) and *C1qa*$^{-/-}$ mice but not in the corresponding serum samples. A representative of three experiments is shown. MM, molecular mass.

on the epithelium. Increased C4 and C3 deposition on the inflammatory middle ear epithelium was observed in wild-type mice after infection. C4 was not present in *C1qa*$^{-/-}$ mice, but C3 deposition was detected on the epithelium. This indicates that complement activation in the middle ear epithelium does not require the classical pathway. In contrast, although C4 was deposited on the middle ear epithelium of *Bf*$^{-/-}$ mice, only weak C3 deposition was observed around the apical region of

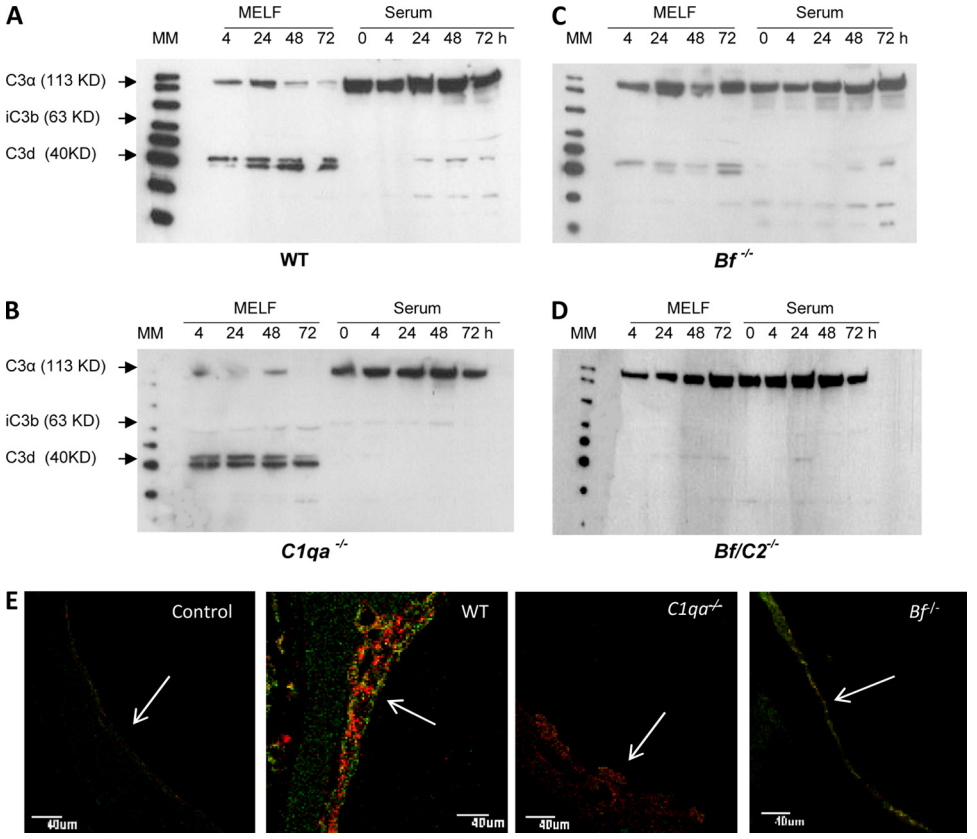

FIG. 3. Complement C3 activation in wild-type (WT) and complement-deficient mice during the course of acute pneumococcal OM. C3 cleavage fragments were analyzed by Western blotting. Approximately 30 μg of protein from the middle ear lavage fluid (MELF) and corresponding serum samples collected and pooled from five mice at 4, 24, 48, and 72 h postinfection was subjected to SDS-PAGE. The immunoreactive bands corresponding to C3α, iC3b, and C3d are denoted. A prominent C3d band was found in the MEFL samples in wild-type (A) and *C1qa*$^{-/-}$ mice (B), whereas weak or faint C3d bands were found in *Bf*$^{-/-}$ (C) and *Bf/C2*$^{-/-}$ mice (D). The smaller bands on the C3 Western blot may be the unidentified degradation products. A representative of three experiments is shown. MM, molecular mass. (E) Representative dual immunofluorescence staining for C3 (red) and C4 (green), and merged results (yellow) in the middle ear mucosa at 24 h postinfection. Faint staining was observed in the sham control of wild-type mice. Increased C3 and C4 immunofluorescence staining in the middle ear epithelium of wild-type mice, increased C3 but invisible C4 immunofluorescence staining in the middle ear epithelium in *C1qa*$^{-/-}$ mice, and increased C4 but weak C3 staining in the apical region of the middle ear epithelium in *Bf*$^{-/-}$ mice are shown. Arrows indicate the middle ear mucosa. Images are representative of three mice in each group examined individually.

the epithelium, suggesting alternative pathway-dependent complement activation on middle ear mucosa. C3 or C4 deposition on the epithelium was not detected in *Bf/C2*$^{-/}$ mice (data not shown). Taken together, the simultaneous increase in factor B and C3 expression, the presence of their fragments in wild-type and *C1qa*$^{-/-}$ mice, and the weak C3 activation in *Bf*$^{-/-}$ mice suggest an essential role for the alternative pathway in the initiation and amplification of complement activation in response to *S. pneumoniae* invasion in the middle ear.

Factor B and C1qa deficiency impedes opsonophagocytosis of *S. pneumoniae* by mouse neutrophils *in vivo*. We previously reported that the recruitment of inflammatory cells is not impaired in complement-deficient mice during pneumococcal OM (27). In the present study, we further characterized these inflammatory cells at 24 h postinfection. Giemsa staining of the cells showed that greater than 98% of cells were neutrophils. In addition, we found that greater than 94% of the inflammatory cells were Ly6G^{+} CD11b^{+} neutrophils (Fig. 4A).

The effects of classical and/or alternative pathway activation on opsonization, attachment, and phagocytosis of *S. pneumoniae* by neutrophils in the mouse middle ear were further evaluated. Confocal fluorescence microscopy of wild-type neutrophils demonstrated more attachment of C3b-opsonized *S. pneumoniae* than that in *C1qa*$^{-/-}$ and *Bf*$^{-/-}$ mice. In contrast, very few C3b-opsonized *S. pneumoniae* bacteria attached to *Bf/C2*$^{-/-}$ neutrophils. Our data confirm that the absence of classical and/or alternative pathway activation greatly diminishes C3b/iC3b/C3c deposition on *S. pneumoniae* and bacterial attachment to neutrophils in the middle ear (Fig. 4B). In addition, colony counts confirmed that there were significantly more *S. pneumoniae* cells attached to and internalized in neutrophils in the middle ears in wild-type than in *C1qa*$^{-/-}$, *Bf*$^{-/-}$, and *Bf/C2*$^{-/-}$ mice (Fig. 4C). Taken together, our data indicate that increased complement opsonins deposited on *S. pneumoniae* via both classical and alternative pathway activation contribute to enhanced phagocytosis by neutrophils during the early stage of OM.

DISCUSSION

Previous studies showed that local expression and synthesis of complement components in the mouse lung and kidney epithelial cells are involved in complement activation in the host innate immune response (2, 24). There are, however, no reports assessing whether *S. pneumoniae* induces complement gene expression in the mouse middle ear epithelium during acute pneumococcal OM. In the current study we found that factor B and C3 gene expression was upregulated in the mouse middle ear epithelium during OM. These findings are analogous to a previous report that showed that factor B and C3 genes are expressed following intratracheal instillation of lipopolysaccharide (LPS) in the mouse lung (2).

It should be noted that the increased C3 concentration in middle ear lavage samples could be attributed to different sources. In addition to middle ear epithelium synthesis, activated neutrophils can synthesize and secrete C3 (4). Increased neutrophil infiltration and higher cytokine levels in complement-deficient mice (27) could account for increased C3 concentrations in the middle ear lavage samples from the infected mice. In addition, vascular transudation of serum-derived complement components might also account for increased C3 in middle ear effusions.

Our current experiments demonstrate that complement alternative pathway and C3 activation in the middle ear account for the local immune response to *S. pneumoniae*. A previous study reported that C3a and C5a levels in human middle ear effusions were much higher than in their corresponding serum samples (16). These findings support the concept that local complement activation is an important modulator of the host defense against otopathogens.

The complement system is activated by three major pathways: classical, alternative, and lectin. The alternative complement pathway contributes significantly to the innate immune response in sepsis (12, 17). In the current study, we found that alternative pathway activation correlated with C3 cleavage in the middle ear lavage samples as well as C3b/iC3b/C3c deposition on *S. pneumoniae*. These findings suggest that the alternative pathway plays a significant role during the initial stage of complement activation in the infected middle ear. In addition, mice with targeted deletion of the factor B and C2 genes demonstrated only native C3 in middle ear lavage and serum samples, suggesting that an intact complement pathway is required for the initiation and propagation of complement activation. Factor B was not detected in *Bf*$^{-/-}$ mice in middle ear lavage and serum samples, but we did find low levels of C3 cleavage fragments in both samples. This might be explained by initial activation of the classical/lectin pathways, followed by amplification by the alternative pathway. However, without alternative pathway amplification, a much lower level of C3 cleavage fragments was observed in the middle ear lavage samples of *Bf*$^{-/-}$ mice than in factor B-sufficient mice.

The mechanisms of activation and regulation of the alternative pathway during pneumococcal OM are not fully understood. It is possible that intact pneumococci or cell constituents may activate the complement system nonspecifically via the alternative pathway. A previous report demonstrated that innate immune receptor agonists Toll-like receptor 4 (TLR4) and TRIF mediate upregulated expression of factor B in macrophages by LPS (11). A further understanding of the complexity of cross talk between the complement pathway activation and other innate immune pathways is needed. Exploring the biological consequences of alternative pathway activation in the host defense against *S. pneumoniae* and in resolution of inflammation during OM would shed new light on the pathogenesis of OM.

S. pneumoniae capsule can impede opsonophagocytosis by several mechanisms. These include inhibiting both the classical and alternative complement pathways and decreasing C3b/iC3b deposition on the bacteria (9). Several mechanisms by which *S. pneumoniae* can evade the complement system have been described. Pneumococcal cell surface proteins such as PspA, for example, interfere with C3 deposition on the pneumococcal cell surface and inhibit complement-mediated opsonization (29). Pneumococcal surface protein C (PspC) binds to complement factor H, a circulating inhibitor of alternative pathway activation (10). Furthermore, a new allelic variant (PspC4.4) of PspC has been identified as a binding ligand for C4BP, the major soluble inhibitor of the classical and lectin pathways (7). In the present study, we observed a close correlation between complement activation and opsonophagocyto-

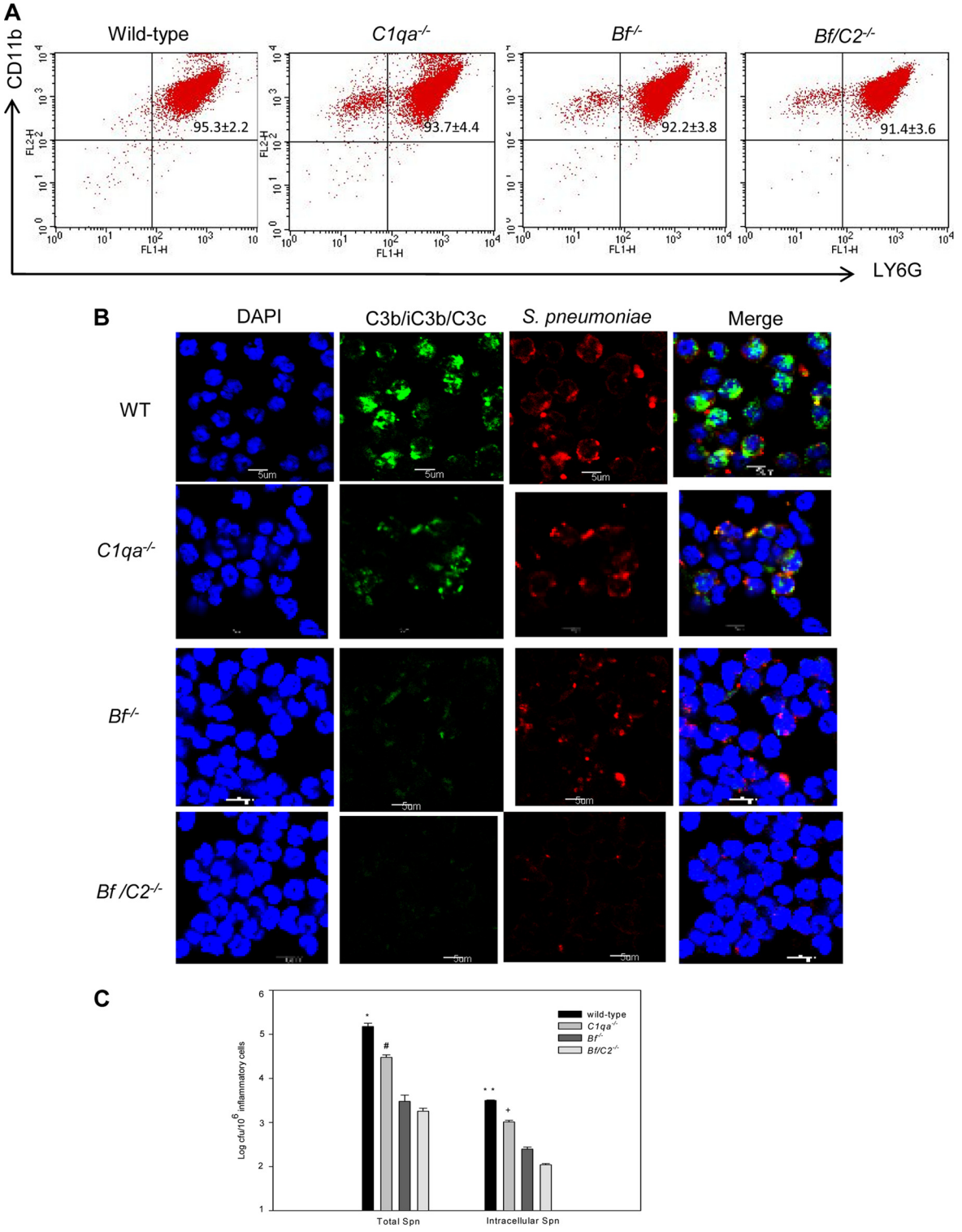

FIG. 4. Opsonophagocytosis of *S. pneumoniae* by the neutrophils in the middle ear at 24 h postinfection. (A) Recruitment of neutrophils into the middle ear at 24 h after *S. pneumoniae* infection. The proportion of $LY6G^+$ $CD11b^+$ granulocytes in the middle ear lavage fluid samples was determined by flow cytometry analysis of inflammatory cells in the middle ear lavage fluid samples from wild-type, $C1qa^{-/-}$, $Bf^{-/-}$, and $Bf/C2^{-/-}$ mice. The numbers in diagram represent the mean percentage of $LY6G^+$ $CD11b^+$ granulocytes (± SEM) from three independent experiments. There were no significant differences between wild-type and the complement-deficient mice. Data from a single set of experiments are shown but are representative of at least three such experiments. (B) Confocal microscope images of opsonophagocytosis of *S. pneumoniae* type 6A by neutrophils in the middle ear of wild-type (WT) and complement-deficient mice. These middle ear lavage fluid samples were immunofluorescently labeled to visualize *S. pneumoniae* (red), C3b/iC3b/C3c (green), and nucleic acids (blue). Bacteria in the merged images may appear as pink-yellow (merge of red and green) or white (merge of red, green, and blue). Images are representative of three independent studies. (C) Quantitative assay for binding and ingestion of *S. pneumoniae* (Spn) by the neutrophils. These results are expressed as the mean CFU count/10^6 neutrophils (± SEM) binding to the neutrophils or ingested by the neutrophils from three different experiments. ∗, $P < 0.001$ compared with values determined for the complement-deficient groups; ∗∗, $^+$, and #, $P < 0.05$ compared with $Bf/C2^{-/-}$ group.

sis. In support of a previous report (30), we found that the opsonophagocytosis of *S. pneumoniae* by neutrophils in the middle ear is largely dependent on C3b/iC3b deposition and was inhibited in $Bf^{-/-}$ or $Bf/C2^{-/-}$ mice. Our data suggest that factor B plays as essential role in promoting *S. pneumoniae* opsonophagocytosis by neutrophils in the middle ear and amplifying host innate immune resistance to *S. pneumoniae* during the course of OM.

We found that C1qa deficiency did not reduce C3 activation but did affect C3b/iC3b/C3c deposition on *S. pneumoniae*. $C1qa^{-/-}$ mice have a reduced ability to clear *S. pneumoniae* compared to wild-type mice (27). The reduced opsonophagocytosis in $C1qa^{-/-}$ mice might be a result of decreased classical pathway activation through an antibody-independent mechanism such as C-reactive protein via C1 binding. However, complement activation by both the classical and alternative pathways is well known to occur in clinical cases of OME (20). In children with relapsing otitis media, specific antibody against pneumococcal polysaccharide forms immune complexes with pneumococcal antigens to activate the classical pathway.

In conclusion, the current study demonstrates that factor B and C3 genes are highly induced in the middle ear epithelium by *S. pneumoniae*. Using complement-deficient mice, we found that C3 activation and *S. pneumoniae* opsonophagocytosis by neutrophils in the middle ear were attenuated in factor B-deficient mice. These findings support the concept that alternative pathway-dependent complement activation in the middle ear is important to protect against acute pneumococcal OM. However, although C1qa deficiency has had less impact on C3 activation, opsonophagocytosis of *S. pneumoniae* by neutrophils in the middle ear was also reduced in $C1qa^{-/-}$ mice. Our data suggest that the classical and alternative pathways may both contribute differently to complement-mediated innate immune mechanisms against acute pneumococcal OM.

ACKNOWLEDGMENTS

This study was supported by award number R01DC009235 from the National Institute on Deafness and Other Communication Disorders (H.H.T.), National Institutes of Health, R01 DK076690 from the National Institute of Diabetes and Digestive and Kidney Diseases (J.M.T.), National Institutes of Health, and HL52886, HL56086, HL099130, AI089781, and HL092469 (G.L.S.) from the National Institutes of Health.

We thank Thomas F. DeMaria and Calvin M. Kunin for their critical review of the manuscript, Marina Botto, Imperial college, London, for her agreement to use $C1qa^{-/-}$ and $Bf/C2^{-/-}$ mice, assistance from the Flow Cytometry Core Lab at Davis Heart and Lung Research Institute, and the Campus Microscopy and Imaging Facility at The Ohio State University.

REFERENCES

1. **Andersson, B., et al.** 1981. Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. Infect. Immun. **32:**311–317.
2. **Bolger, M. S., et al.** 2007. Complement levels and activity in the normal and LPS-injured lung. Am. J. Physiol. Lung Cell. Mol. Physiol. **292:**L748–L759.
3. **Botto, M., et al.** 1998. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat. Genet. **19:**56.
4. **Botto, M., D. Lissandrini, C. Sorio, and M. J. Walport.** 1992. Biosynthesis and secretion of complement component (C3) by activated human polymorphonuclear leukocytes. J. Immunol. **149:**1348–1355.
5. **Brown, J. S., et al.** 2002. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. Proc. Natl. Acad. Sci. U. S. A. **99:**16969–16974.
6. **Casey, J. R., D. G. Adlowitz, and M. E. Pichichero.** 2010. New patterns in the otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. Pediatr. Infect. Dis. J. **29:**304–309.
7. **Dieudonné-Vatran, A., et al.** 2009. Clinical isolates of *Streptococcus pneumoniae* bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. J. Immunol. **182:**7865–7877.
8. **Hunter, L. L., R. H. Margolis, and G. S. Giebink.** 1994. Identification of hearing loss in children with otitis media. Ann. Otol. Rhinol. Laryngol. Suppl. **163:**59–61.
9. **Hyams, C., E. Camberlein, J. M. Cohen, K. Bax, and J. S. Brown.** 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. Infect. Immun. **78:**704–715.
10. **Jarva, H., et al.** 2002. *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the *pspC* locus-encoded Hic protein that binds to short consensus repeats 8–11 of factor H. J. Immunol. **168:**1886–1894.
11. **Kaczorowski, D. J., et al.** 2010. Pivotal advance: The pattern recognition receptor ligands lipopolysaccharide and polyinosine-polycytidylic acid stimulate factor B synthesis by the macrophage through distinct but overlapping mechanisms. J. Leukoc. Biol. **88:**609–618.
12. **Kerr, A. R., G. K. Paterson, A. Riboldi-Tunnicliffe, and T. J. Mitchell.** 2005. Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. Infect. Immun. **73:**4245–4252.
13. **Long, J. P., H. H. Tong, P. A. Shannon, and T. F. DeMaria.** 2003. Differential expression of cytokine genes and inducible nitric oxide synthase induced by opacity phenotype variants of *Streptococcus pneumoniae* during acute otitis media in the rat. Infect. Immun. **71:**5531–5540.
14. **MacArthur, C. J., et al.** 2006. Evaluation of the mouse model for acute otitis media. Hear. Res. **219:**12–23.
15. **Matsumoto, M., et al.** 1997. Abrogation of the alternative complement pathway by targeted deletion of murine factor B. Proc. Natl. Acad. Sci. U. S. A. **94:**8720–8725.
16. **Mogi, G., and J. M. Bernstein.** 1987. Immune mechanisms in otitis media with effusion, p. 279–299. *In* J. M. Bernstein and P. L. Ogra (ed.), Immunology of the ear. Raven, New York, NY.
17. **Mueller-Ortiz, S. L., S. M. Drouin, and R. A. Wetsel.** 2004. The alternative activation pathway and complement component C3 are critical for a protective immune response against *Pseudomonas aeruginosa* in a murine model of pneumonia. Infect. Immun. **72:**2899–2906.
18. **Närkiö-Mäkelä, M., J. Jero, and S. Meri.** 1999. Complement activation and expression of membrane regulators in the middle ear mucosa in otitis media with effusion. Clin. Exp. Immunol. **116:**401–409.
19. **Närkiö-Mäkelä, M., and S. Meri.** 2001. Cytolytic complement activity in otitis media with effusion. Clin. Exp. Immunol. **124:**369–376.
20. **Prellner, K., and N. I. Nilsson.** 1982. Complement aberrations in serum from children with otitis due to *S. pneumoniae* or *H. influenzae*. Acta Otolaryngol. **94:**275–282.
21. **Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris.** 2010. Complement: a key system for immune surveillance and homeostasis. Nat. Immunol. **11:**785–797.
22. **Rothman, B. L., A. W. Despins, and D. L. Kreutzer.** 1990. Cytokine regulation of C3 and C5 production by the human type II pneumocyte cell line, A549. J. Immunol. **145:**592–598.
23. **Sabharwal, V., S. Ram, M. Figueira, I. H. Park, and S. I. Pelton.** 2009. Role of complement in host defense against pneumococcal otitis media. Infect. Immun. **77:**1121–1127.
24. **Sheerin, N. S., et al.** 2008. Synthesis of complement protein C3 in the kidney is an important mediator of local tissue injury. FASEB J. **22:**1065–1072.
25. **Stockley, R. A., M. Mistry, A. R. Bradwell, and D. D. Burnett.** 1979. A study of plasma proteins in the sol phase of sputum from patients with chronic bronchitis. Thorax **34:**777–782.
26. **Taylor, P. R., et al.** 1998. A targeted disruption of the murine complement factor B gene resulting in loss of expression of three genes in close proximity, factor B, C2, and D17H6S45. J. Biol. Chem. **273:**1699.
27. **Tong, H. H., Y. X. Li, G. L. Stahl, and J. M. Thurman.** 2010. Enhanced susceptibility to acute pneumococcal otitis media in mice deficient in complement C1qa, factor B, and factor B/C2. Infect. Immun. **78:**976–983.
28. **Tong, H. H., J. P. Long, D. Li, and T. F. DeMaria.** 2004. Alteration of gene expression in human middle ear epithelial cells induced by influenza A virus and its implication for the pathogenesis of otitis media. Microb. Pathog. **37:**193–204.
29. **Tu, A. H., R. L. Fulgham, M. A. McCrory, D. E. Briles, and A. J. Szalai.** 1999. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect. Immun. **67:**4720–4724.
30. **Xu, Y., et al.** 2001. Complement activation in factor D-deficient mice. Proc. Natl. Acad. Sci. U. S. A. **98:**14577–14582.

Editor: A. Camilli