Complement component 5 contributes to poor disease outcome in humans and mice with pneumococcal meningitis

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Pneumococcal meningitis is the most common and severe form of bacterial meningitis. Fatality rates are substantial, and long-term sequelae develop in about half of survivors. Disease outcome has been related to the severity of the proinflammatory response in the subarachnoid space. The complement system, which mediates key inflammatory processes, has been implicated as a modulator of pneumococcal meningitis disease severity in animal studies. Additionally, SNPs in genes encoding complement pathway proteins have been linked to susceptibility to pneumococcal infection, although no associations with disease severity or outcome have been established. Here, we have performed a robust prospective nationwide genetic association study in patients with bacterial meningitis and found that a common nonsynonymous complement component 5 (C5) SNP (rs17611) is associated with unfavorable disease outcome. C5 fragment levels in cerebrospinal fluid (CSF) of patients with bacterial meningitis correlated with several clinical indicators of poor prognosis. Consistent with these human data, C5a receptor–deficient mice with pneumococcal meningitis had lower CSF wbc counts and decreased brain damage compared with WT mice. Adjuvant treatment with C5-specific monoclonal antibodies prevented death in all mice with pneumococcal meningitis. Thus, our results suggest C5-specific monoclonal antibodies could be a promising new antiinflammatory adjuvant therapy for pneumococcal meningitis.

Introduction

Community-acquired bacterial meningitis continues to exact a heavy toll, even in developed countries, despite the implementation of childhood vaccination programs and effective antimicrobial agents (1, 2). The most common etiologic agents of bacterial meningitis are Streptococcus pneumoniae and Neisseria meningitidis, with the first bacterium responsible for two-thirds of cases in Europe and the United States (1). The fatality rates in patients with meningitis caused by these microorganisms are substantial, at 26% and 9%, respectively (3), and long-term sequelae, including hearing loss, focal neurological deficit, and cognitive impairment, develop in about half of survivors (1).

Experimental animal models have shown that outcome in bacterial meningitis is related to the severity of inflammation in the subarachnoid space, and it was suggested that outcome could be improved by modulation of the inflammatory response, for example, with dexamethasone (4). Many randomized clinical trials of dexamethasone in bacterial meningitis have been performed, but results have remained ambiguous (5–8). An individual patient data meta-analysis of 5 large recent trials showed no effect of dexamethasone (7). A prospective cohort study showed a decrease in mortality from 30% to 20% in adults with pneumococcal meningitis after nationwide implementation of dexamethasone in the Netherlands (9). New adjunctive therapies are needed to improve the prognosis of bacterial meningitis.

Genetic association studies may reveal new targets for adjuvant therapies (10). Genetic defects in the complement system have been studied in patients with extreme phenotypes of meningitis, particularly those with familial or recurrent disease, focusing on susceptibility to invasive pneumococcal and meningococcal disease (11). The complement system can be divided into 3 activation pathways (the classical, lectin, and alternative pathways), which all converge on a common terminal pathway (12). An essential step in the classical and lectin pathways is cleavage of complement component C2 into its fragments, C2a and C2b. A retrospective study, including 40,000 patients with suspected complement deficiency, identified 40 individuals with C2 deficiency due to a 28-bp deletion (13). A history of invasive infections, mainly pneumococcal infections, was found in 23 (58%) of these individuals (13). The formation of the alternative pathway C3 convertase complex (C3bBb) is a crucial step in the alternative pathway and requires complement factor D (F D) (12). F D deficiency due to uncommon SNPs has been described in cases and families with meningococcal and pneumococcal infections (14, 15). C3bBb is stabilized by properdin (16), and properdin deficiency predisposes to meningococcal disease due to serogroups W135 and Y; one-third of patients with meningococcal disease caused by these serotypes are properdin deficient. The common terminal pathway consists of complement components C5–C9, and activation forms the anaphylatoxin C5a, a strong proinflam-
complement components have been recognized as a cause of recurrent disease (12). Deficiencies in these late components recognition molecule activates the lectin pathway upon binding to microorganisms (12). Factor H (fH) regulates the alternative pathway by inactivating C3bBb (12). The fH –496C/C genotype was found to be associated with meningococcal disease (OR, 2.0; 95% CI, 1.3–3.2) (17). Most of the candidate gene approach studies lacked power to detect true associations (11). Recently, a genome-wide association study (GWAS) on host susceptibility to meningococcal disease identified a locus in the complement factor H (CFH) region, providing the first convincing evidence for a role of SNPs in complement genes in susceptibility to infections (18). Little is known about the role of complement SNPs in bacterial meningitis, and so far no associations with disease severity or outcome have been reported in case-control studies for complement SNPs or GWAS (11).

Studies in animal models have provided evidence for involvement of the complement system in modulating severity of pneumococcal meningitis. In rabbits depleted of C3 by administering cobra venom factor, intracisternal inoculation of S. pneumoniae resulted in higher bacterial titers in the cerebrospinal fluid (CSF) than in complement-sufficient control animals (19). Other studies showed an increased pneumococcal outgrowth in the brain and blood in gene-targeted mice lacking C1q, affecting only the classical pathway; C3, affecting all complement activation pathways; or the receptor for the opsonin C3b/iC3b (CR3) (20, 21). C3 deficiency led to diminished brain inflammation, paralleled by an attenuation of intracranial complications. However, the lack of CR3-mediated opsonophagocytosis resulted in increased bacteremia that worsened outcome. These data provide evidence that the complement system is important in bacterial meningitis and that antagonizing the detrimental proinflammatory effects of the complement system without inhibiting its antimicrobial activity might be a promising adjuvant therapy option.

We performed a prospective nationwide genetic association study in patients with community-acquired bacterial meningitis to investigate the roles of common genetic variants in the complement system in outcome. By analyzing clinical data and CSF, we identified the potential impact and functionality of a SNP that was associated with outcome. We then validated and explored our findings in an animal model of pneumococcal meningitis and investigated whether adjuvant treatment with a monoclonal antibody targeted against this specific complement component could improve outcome.

### Results

In a prospective nationwide cohort study, we included 642 out of 762 (84%) identified episodes of community-acquired CSF culture-proven bacterial meningitis in 636 patients. The distribution of causative bacteria was S. pneumoniae in 468 (73%), N. meningitidis in 80 (13%), and other bacteria in 94 (15%) episodes. DNA samples were obtained from 439 patients (68%) and 302 controls. Controls were patients’ partners or nonrelated proxies living in the same dwelling, as household members they had similar exposure to bacteria through nasopharyngeal colonization, and were matched for age, ethnicity, and sex (ref. 22 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI57522DS1). Predisposing conditions, most commonly otitis media or sinusitis (36%) and immunocompromised state (22%), were present in 58% of episodes (Table 1). In 13% of episodes, patients were comatose on admission, and 32% of the episodes had focal neurologic deficits. The case fatality rate was 8%, and 24% of the episodes had an unfavorable outcome, defined as a score of 1 through 4 on the Glasgow Outcome Scale (GOS) (23). Patients for whom DNA was obtained were on average...
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For favorable and unfavorable outcome, the number of patients in each allele or genotype is listed. *Control population did not comply with HWE.

Youth older than 18 years and with less severe disease than patients for whom DNA was not obtained (Supplemental Table 2).

Genetic association study on common variants in the complement system.
We selected all SNPs with a minor allele frequency of more than 5% in genes coding for complement components (C1QA, C1QB, C1QC, C2, C3, C5, C6, C7, C8B, C9, CFD, CFH, CFI, and CFP) for which a commercial genotyping assay was available. A total of 17 SNPs were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems). The genotyping success rate was more than 95% for all assays. In 16 out of 17 assays, genotype frequency of controls of patients of mixed European descent concurred with the Hardy-Weinberg equilibrium (HWE; Supplemental Table 3). We compared the genotype frequency of patients with a favorable outcome, defined as a GOS of 3 or better, and those without DNA available.

Table 2
Genotyping analysis of 17 common complement component polymorphisms in 329 patients with bacterial meningitis with favorable outcome and 105 with unfavorable outcome

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>Favorable outcome</th>
<th>Unfavorable outcome</th>
<th>Risk allele or genotype</th>
<th>OR</th>
<th>P</th>
</tr>
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<tr>
<td>C3</td>
<td>rs1047286</td>
<td>543</td>
<td>111 218 107 2</td>
<td>169 37 69 31 3</td>
<td>BB</td>
<td>4.88 (0.80–29.6)</td>
</tr>
<tr>
<td>C3</td>
<td>rs2230199</td>
<td>539</td>
<td>115 218 103 6</td>
<td>171 33 73 25 4</td>
<td>BB</td>
<td>2.18 (0.60–7.90)</td>
</tr>
<tr>
<td>C5</td>
<td>rs17611</td>
<td>292</td>
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<td>BB</td>
<td>1.70 (1.08–2.67)</td>
</tr>
<tr>
<td>C6</td>
<td>rs1801033</td>
<td>422</td>
<td>232 140 142 45</td>
<td>137 67 45 47 10</td>
<td>A</td>
<td>1.47 (0.73–0.03)</td>
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<tr>
<td>C7</td>
<td>rs1063499</td>
<td>257</td>
<td>401 57 143 129</td>
<td>90 114 23 44 35</td>
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<td>1.39 (0.81–2.40)</td>
</tr>
<tr>
<td>C7</td>
<td>rs13157656</td>
<td>174</td>
<td>472 12 150 161</td>
<td>49 151 5 39 56</td>
<td>BB</td>
<td>1.28 (0.82–2.01)</td>
</tr>
<tr>
<td>C7</td>
<td>rs60714178</td>
<td>91</td>
<td>565 8 75 245</td>
<td>34 176 5 24 76</td>
<td>AA</td>
<td>2.00 (0.64–6.25)</td>
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<tr>
<td>CBBB</td>
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<td>35</td>
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<td>1.02 (0.99–1.05)</td>
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<td>110 78 32 46 16</td>
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<td>2.11 (0.95–2.16)</td>
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<td>1.52 (0.74–3.13)</td>
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<td>32 174 2 28 73</td>
<td>BB</td>
<td>1.26 (0.26–6.02)</td>
</tr>
<tr>
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<td>2.23 (1.07–4.65)</td>
</tr>
<tr>
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<td>434</td>
<td>224 148 138 43</td>
<td>137 73 50 37 18</td>
<td>BB</td>
<td>1.38 (0.76–2.51)</td>
</tr>
</tbody>
</table>

For favorable and unfavorable outcome, the number of patients in each allele or genotype is listed. *Control population did not comply with HWE.
different mutants. Components of the complement system are known to modulate inflammatory responses (12, 26).

First, we compared mice with a deficiency of the C5a receptor (C5ar1–/– mice) to WT mice. CSF wbc count in infected C5ar1–/– mice was decreased to 25% of that in WT mice (Figure 3). The reduced inflammatory response in C5ar1–/– mice was associated with better clinical status (clinical score shown in Figure 3), with less severe hypothermia, reduced weight loss, and conserved exploratory behavior in the open-field test (OFT) (C5ar1–/– vs. WT, body temperature, 37.3°C ± 0.42°C vs. 36.6°C ± 0.54°C, P = 0.004; weight loss, 11.1% ± 1.58% vs. 13.3% ± 2.38%, P = 0.031; OFT, 23 ± 2 fields vs. 3 ± 4 fields, P = 0.019).

A strong granulocytic inflammatory response contributes substantially to neuropathology in pneumococcal meningitis (27); this was supported by our finding that granulocyte depletion was protective against meningitis-related brain damage (Supplemental Figure 1). Therefore, we evaluated major meningitis-associated intracranial complications in our model: raised intracranial pressure (ICP), decreased blood-brain barrier (BBB) integrity, and intracerebral hemorrhages. BBB breakdown (brain albumin content) was combined with the number of intracerebral hemorrhages to obtain the neuroscore, as described previously (25).

C5ar1–/– mice had reduced ICP and lower neuroscores when compared with those of WT mice (Figure 3). There was no difference in cerebellar bacterial titer (6.58 ± 0.59 log10 CFU/cerebellum in WT mice vs. 6.15 ± 0.73 log10 CFU/cerebellum in C5ar1–/– mice) or mortality rate (0 out of 10 vs. 0 out of 9 for WT vs. C5ar1–/– mice) between C5ar1–/– and WT mice. The altered recruitment of CSF inflammatory cells in C5ar1–/– mice prompted us to analyze the levels of cytokines and inflammatory mediators in mouse brain homogenates. Amounts of IL-6 (data not shown), CXCL1/KC, and CXCL2/MIP-2 were all reduced in infected C5ar1–/– mice compared with those in infected WT mice (Figure 3).

In order to evaluate whether the decreased CSF wbc count observed in the C5ar1–/– mice was mediated through chemokine regulation by C5a, infected animals were treated with anti-CXCL2/ MIP-2 antibodies, either alone or in combination with anti-CXCL1/ KC antibodies. Treatment with anti-CXCL2/MIP-2 antibodies alone reduced CSF wbc count by 40% (not significant), whereas, when combined with anti-CXCL1, treatment caused a reduction of CSF wbc count by 63% (P = 0.003). The animals treated with both CXCL1 and CXCL2 antibodies were in a better clinical state compared with that of the untreated mice (Supplemental Table 5).

To analyze the role of the MAC, we investigated mice with a mutation in the complement component 6 gene (C6–/– mice), which are unable to form the MAC, and mice gene deleted for CD59 (Cd59a–/– mice), the in vivo inhibitor of the MAC (12). C6–/– mice with pneumococcal meningitis tended to have lower CSF wbc counts as compared with those of WT mice (Supplemental Figure 2), whereas Cd59a–/– mice had increased CSF wbc counts compared with those of WT animals (Supplemental Figure 3). No differences were detected between C6–/–, Cd59a–/–, and WT mice in clinical scores, ICP, or neuroscores (Supplemental Figure 3). However, the mortality rate among C6–/– mice was higher (7 out of 14 [50%]) compared with that of Cd59a–/– mice (2 out of 11 [18%]) and WT mice (2 out of 20 [10%]). This difference was attributable to a more severe damage of the BBB in C6–/– mice compared with that of WT mice (brain albumin content, 487.0 ± 287.7 ng/μg vs. 242.7 ± 178.0 ng/μg, P = 0.014). Levels of IL-6 and CXCL2/MIP-2 were similar among the 3 mouse strains.

We next investigated the role of C3a in pneumococcal meningitis. The anaphylatoxin C3a has been shown to be involved in immune regulation of inflammatory CNS diseases (28), and we previously described increased expression of the C3a receptor in mice with pneumococcal meningitis (21). Mice deficient in the C3a receptor (C3ar1–/– mice) and mice expressing C3a exclusively

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP ID</th>
<th>Favorable outcome</th>
<th>Unfavorable outcome</th>
<th>Risk allele or genotype</th>
<th>OR (95% CI)</th>
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<td>C3</td>
<td>rs1047286</td>
<td>354 76 141 72 2</td>
<td>127 33 50 27 3</td>
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<td>4.15 (0.68–25.3)</td>
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<td>C3</td>
<td>rs2230199</td>
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<td>129 29 54 21 4</td>
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<td>2.24 (0.59–8.56)</td>
</tr>
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<td>57 107 14 29 39</td>
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<td>1.40 (0.61–3.19)</td>
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<td>BB</td>
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</tr>
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<td>109 49 37 35 7</td>
<td>A</td>
<td>1.08 (0.46–2.52)</td>
</tr>
<tr>
<td>CFH</td>
<td>rs1065489</td>
<td>68 362 10 48 157</td>
<td>30 130 4 22 54</td>
<td>A</td>
<td>1.26 (0.72–2.19)</td>
</tr>
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<td>CFH</td>
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<td>108 56 39 30 13</td>
<td>BB</td>
<td>1.04 (0.52–2.08)</td>
</tr>
</tbody>
</table>

For favorable and unfavorable outcome, the number of patients in each allele or genotype is listed. *Control population did not comply with HWE.
in the CNS using the GFAP promoter (C3a/GFAP mice) were compared with infected WT mice. C3a/GFAP mice had increased CSF wbc counts as compared with those of WT and C3ar1−/− mice (Supplemental Figure 3), but other parameters were similar (clinical scores, ICP, neuroscores, proinflammatory mediators, and cytokines; Supplemental Figure 3).

**Adjuvant treatment with C5 antibody.** The experiments performed with C3ar1−/− mice suggested a major role for C5a in the regulation of the immune response in pneumococcal meningitis; we therefore evaluated treatment with a neutralizing monoclonal antibody directed against murine C5 (C5-Ab, BB5.1) in the model. Animals were given i.p. C5-Ab or i.p. IgG (1 mg per mouse, each) prior to infection. Levels of cerebral sC5b-9 were significantly reduced in animals treated with C5-Ab (Figure 4). Consistent with that in C3ar1−/− mice, WT mice treated with C5-Ab prior to infection displayed a reduced CSF wbc count accompanied by better clinical scores when compared with those of animals treated with mouse IgG (Figure 4).

To define the site of action of C5 neutralization, we applied a low dose of C5-Ab (30 μg per mouse) to infected mice either by the i.p. (systemic) or intrathecal (i.t.) (local) route. Mice treated i.t. with C5-Ab had lower CSF leukocyte counts, less meningitis-associated intracranial complication, and better clinical status, as compared with mice treated with control IgG (Supplemental Table 5). No difference between infected mice treated with i.p. C5-Ab or control IgG was observed.

We next compared adjunctive C5-Ab treatment with adjunctive treatment with dexamethasone, the standard adjunct in humans with pneumococcal meningitis, (1, 8), or adjunctive treatment with neutralizing TLR2 and TLR4 antibodies. Treatment with TLR2 and TLR4 antibodies was based on our recent observation that TLR2 and TLR4 are essential in mounting the CNS innate immune response in pneumococcal meningitis (29). All adjunctive therapies were administered i.p. 24 hours after infection concomitant with antibiotic treatment consisting of ceftriaxone. In these experiments, i.p. treatment with PBS or IgG served as control.
Treatment with the C5-Ab prevented lethal outcome in all treated animals, as shown by a significant decrease in the mortality rate as compared with treatment with IgG (deaths, 7 out of 21 mice [33%]; Figure 5A). Adjunctive treatment with dexamethasone reduced the mortality rate as compared with that with PBS (deaths, 2 out of 10 mice [20%] vs. 5 out of 16 mice [31%]) but was less effective when compared with treatment with C5-Ab (Figure 5C). Adjunctive treatment with anti-TLR2 and TLR4 antibodies caused a significant attenuation of meningeal inflammation and brain tissue damage, in line with our previous study (29) (Supplemental Table 5); however, these antibodies had no effect on mortality (deaths, 2 out of 8 mice [25%]; Figure 5B). Adjunctive treatment with C5-Ab, but not with dexamethasone or anti-TLR2 and 4 antibodies, resulted in a reduction of meningitis-induced brain damage (neuroscores, 2.3 ± 1.6 vs. 4.2 ± 1.6 in IgG-treated mice [P = 0.012], vs. 4.3 ± 1.5 in anti-TLR2 − and TLR4−treated mice, 3.5 ± 2.0 in dexamethasone-treated mice, and 3.7 ± 1.7 in PBS-treated mice).

Discussion
We demonstrated that a common variant in C5 was associated with unfavorable outcome in adults with community-acquired pneumococcal meningitis. The anaphylatoxin C5a was identified as the crucial complement product in pneumococcal meningitis. Neutralization experiments showed that adjunctive treatment with C5-Ab improved outcome in mice with pneumococcal meningitis. The observed effect of C5-Ab was superior to that of adjuvant dexamethasone, the antiinflammatory drug that is currently recommended in clinical guidelines (2, 30). Since anti-C5 antibodies are currently licensed for clinical use (eculizumab) or used in clinical trials (pexelizumab) (31, 32), our results present a promising treatment option for future patients with community-acquired bacterial meningitis.

Patients with the rs17611 GG genotype were at higher risk for unfavorable outcome as compared with carriers of the A allele (OR, 2.26; 95% CI, 1.30–3.94). Our genetic association study was nationwide, and, therefore, we were able to study a representative sample of adults with acute bacterial meningitis. The prospective approach allowed us to collect comprehensive clinical data, resulting in a well-defined group of patients with microbiologically confirmed community-acquired bacterial meningitis. Our large sample gave us the statistical power to perform a Bonferroni correction for multiple testing, and, subsequently, we were able to validate our findings in a mouse model of pneumococcal meningitis.

Patients with the rs17611 risk genotype GG had lower CSF wbc counts on admission. Clinical studies have shown that lower CSF wbc counts on admission in patients with bacterial meningitis are associated with sepsis and systemic compromise and adverse outcomes later in disease course (3, 33). Sepsis was not more common in patients with the GG genotype in this study, although power may be insufficient to detect such a difference. Animals studies in a pneumococcal meningitis model showed that lower CSF wbc counts early in disease course were associated with high bacterial load, which correlates with intracranial complications and poor outcome (34). These experiments also showed that lower in disease course, higher CSF wbc counts correlated with high bacterial loads and were associated with poor outcome (34). Other experimental work in pneumococcal meningitis showed a critical role for the cumulative exposure to bacteria during the infection period (35). We speculate that the lower CSF wbc counts in patients with the risk genotype may be due to a reduced chemoattractant function of C5a.

Functional studies have previously shown that SNPs in complement factors can influence complement activation and binding affinity independent of concentration (36, 37). A study on rs17611 function showed the GG genotype was associated with lower serum C5 concentration among 100 healthy volunteers (38). A follow-up study, however, showed that these subjects had serum C5 activity similar to that of those with rs17611 AA/AG, despite lower C5 serum concentration (39). This observation is consistent with our results, which showed similar C5a and TCC concentration in both genotypes.

The anaphylatoxin C5a is a powerful chemoattractant, guiding neutrophils but also directly stimulating the production of cytokines, chemokines, and adhesion molecules (12, 40). Major neurologic complications in patients with pneumococcal meningitis include cerebrovascular complications and brain edema, which are caused, at least partly, by massive neutrophil inflammatory reaction. In patients with bacterial meningitis, CSF C5a concentrations were markedly elevated, and C5a levels were associated with high CSF wbc counts and unfavorable outcome. In our
mouse model, deficiency of the receptor for C5a led to an improved clinical status and clinical course. C5a receptor deficiency and C5 neutralization resulted in a marked reduction of CSF wbc counts in the pneumococcal mouse model, with lower concentrations of IL-6, CXCL1, and CXCL2 in C5ar1−/− mice. Pretreatment with CXCL1 and CXCL2 antibodies caused a reduction of CSF wbc count, but to a lesser extent than that found in C5ar1−/− mice, indicating that C5a regulates chemokine expression but also has a direct chemotactic effect. In our experiments, i.t. anti-C5 treatment also led to a significant reduction in CSF pleocytosis. Previous work showed that treatment with antibodies to native human C5 inhibited leukocyte influx in rabbits with pneumococcal meningitis (40), and intracisternal administration of C5a caused rapid influx of wbc into the CSF of rabbits (41). C5a-mediated neutrophilic inflammation may cause direct tissue injury by release of cytotoxic products from neutrophils and/or by precipitating cerebral vasculitis and a subsequent reduction in blood supply to the brain (27). This concept is supported by evidence presented here and in previous studies demonstrating that neutrophil depletion approaches are beneficial in pneumococcal meningitis, particularly when used as adjunctive treatment with antibiotic therapy (27, 42). These data seem to contradict our observation in humans that the rs17611 risk genotype GG had lower CSF wbc count; however, CSF wbc counts were determined in samples withdrawn on admission, early in the course of the disease. Bacterial titers were not determined in our patients; nevertheless, it is noteworthy that the inoculum size does not correlate with subsequent bacterial titers but does determine the disease kinetics. As a consequence, the precise classification of disease stage is not possible in patients with pneumococcal meningitis. The role of C5a is not limited to its chemoattractant and pro-inflammatory function. First, C5a can induce the expression of tissue factor and plasminogen activator inhibitor-1, leading to amplification of coagulation and inhibition of fibrinolysis (43, 44). The relation between C5a and coagulation pathways is reciprocal: thrombin directly cleaves C5 and generates active C5a, and thrombin-activatable carboxypeptidase B inhibits C5a (43, 45). The procoagulant activity of C5a may represent an additional and/or additive factor in the vascular occlusion process in bacterial meningitis (24, 46, 47). Second, C5a increases vascular permeability, thereby contributing to meningitis-induced brain edema.
In our experiments, C5a receptor deficiency and C5 neutralization resulted in a reduction of brain albumin concentrations, indicative of a protective effect against meningitis-induced BBB breakdown. In line with this finding is the recent observation that C5a receptor inhibition maintained the integrity of the BBB in experimental lupus (48). Moreover, silencing of the C5ar1 gene with siRNA was found to prevent the bacterial lipopolysaccharide-induced increased vascular permeability in multiple organs (49).

Finally, very high concentrations of C5a were shown to induce rapid apoptosis in neuronal cells via neuronal C5a receptor–associated signal transduction pathways (50), whereas in lower concentrations, C5a inhibited apoptosis, induced neuroproliferation, and decreased glutamate excitotoxicity (51). These findings imply that C5a may function as a direct modulator of brain tissue injury in pneumococcal meningitis.

Adjunctive treatment with C5-Ab resulted in a reduction in meningitis-induced brain damage and prevented death, despite having no effect on either bacterial outgrowth in the CSF and blood or antibiotic-induced bacterial killing in experimental pneumococcal meningitis. Complement-mediated opsonophagocytosis and not MAC-mediated bacterial lysis is the major host defense mechanism against invasive pneumococcal infections. In contrast, MAC is known to play a major role in meningococcal killing. Anti-C5 antibodies that block C5a and MAC formation were found to interfere with bacterial lysis using a human whole blood model of meningococcal sepsis (52). However, this study also showed that C5a-specific antibodies (monoclonal antibody 137-126) can bind the C5a moiety and inhibit the harmful effects of C5a while preserving MAC-mediated bacterial killing (52).

The observed adjuvant effect of C5-Ab was superior to that of neutralizing antibodies against TLR2 and TLR4, 2 pattern recognition receptors (PRRs) that have been shown to be essential for mounting the innate immune response to pneumococcal infection of the CNS in experiments using Tlr2−/−, Tlr4−/−, and Tlr2/4−/− mice (27, 29). Indeed, in our neutralization experiments, antibodies to both TLR2 and TLR4, when administered prior to infection, produced a similar phenotype to that seen in the receptor-deficient animals with reduced CSF pleocytosis and improved brain pathology. Our data show that TLR signaling is vital for the initial innate immune response but dispensable for the maintenance of inflammation in meningitis during the later disease course. The presence of S. pneumoniae in the subarachnoid space is initially recognized by TLR2 and TLR4 as well as other PRRs. Activation of TLR2 and TLR4 by pneumococci leads to MyD88-dependent induction and activation of the complement system in the brain (29). Among the complement components produced, C5 and its activation product C5a have now been singled out to be crucial for the propagation of the inflammatory reaction. The C5a-driven inflammatory reaction, in turn, contributes substantially to meningitis-induced vascular and tissue injury, thus representing a major determinant for the outcome of the disease.

Our study has some limitations. A selection bias was introduced since DNA was not available for a considerable proportion of patients (32%), particularly those with more severe disease. Inclu-
sion of patients with less severe disease will decrease study power, resulting in type II errors. However, this will not negate the association of rs17611 with outcome. The nationwide design allowed us to detect this selection bias (11, 53). Furthermore, there may be functional differences between the complement systems of humans and mice. Animal models in rheumatoid arthritis showed a beneficial effect of C5a receptor blockade, but a clinical trial showed no benefit (54, 55). However, bacterial opsonization by mouse complement is known to be similar to the human situation (56). Therefore, we believe that our model is valid and provides valuable information on complement function in pneumococcal meningitis. Overall, we have used a clinical-based approach to generate a hypothesis that was subsequently confirmed in animal studies.

Methods

Dutch bacterial meningitis cohort
The nationwide prospective cohort study included patients with bacterial meningitis that were older than 16 years of age with positive CSF cultures, who were identified by NRLBM from March 2006 to June 2009. NRLBM provided the names of the hospitals in which patients with bacterial meningitis had been admitted 2–6 days previously, and the treating physician was contacted for permission to include the patient. Controls for exposure/susceptibility were patients’ partners or their nonrelated proxies living in the same dwelling. Data on age, sex, and ethnicity of controls were collected. Secured online case-record forms were used to collect data on patient history, symptoms and signs on admission, treatment, complications, and outcome. Data was graded at discharge according to the GO5, a well-validated instrument with good inter-observer agreement (23). A score of 1 on this scale indicates death; a score of 2 indicates a vegetative state; a score of 3 indicates severe disability; a score of 4 indicates moderate disability; and a score of 5 indicates mild or no disability. A favorable outcome was defined as a score of 5, and an unfavorable outcome was defined as a score of 1 to 4. Blood from patients and controls for DNA extraction was collected in sodium/EDTA. DNA was isolated with the Gentra Puregene Isolation Kit (Qiagen), and quality control procedures were performed to determine the yield and purity.

Genotyping
A total of 17 common SNPs in the complement system were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems) with 96 × 96 Dynamic Arrays (Fluidigm) by Service XS, Leiden, the Netherlands, and the Genetics Core Facility in the Academic Medical Center. Laboratory personnel were blinded to clinical information.

CSF complement analysis
CSF of patients was obtained from the diagnostic lumbar puncture. Subsequently, CSF and WBC were stored separately at −80°C. CSF complement analysis

Animal pneumococcal meningitis model
A well-characterized mouse model of pneumococcal meningitis was used in this study (25). Prior to infection, mice were weighed and scored clinically, and temperature was taken. For clinical scoring, different tasks were evaluated, namely a postural reflex test and a beam walk test. Additionally, clinical scoring comprised presence of seizures, piloerection, or reduced vigilance (57). The maximum clinical score was 12 and indicated severe disease, whereas a score of 0 defined healthy, uninfected mice. To further evaluate locomotor and exploratory behavior the OFT was used. In this test, mice were put in the center of a square box, subdivided into 9 fields. Mice were observed for 2 minutes, and the number of entered fields was counted. After clinical evaluation, bacterial meningitis was induced by intracisternal injection of 15 μl 10⁷ CFUs per ml S. pneumoniae type 2 (D39 strain; provided by Sven Hammerschmidt, University of Greifswald, Greifswald, Germany) under short-term anesthesia with isoflurane. To evaluate the acute disease, animals were investigated 24 hours after infection. To evaluate adjuvant treatment options, mice received antibiotic therapy (100 mg/kg ceftriaxone i.p.) together with adjuvant treatment at 24 hours after infection and were investigated 48 hours after infection. In both settings, at the end of each experiment, animals were weighed and scored clinically as described above, and the temperature was taken. Mice were then anesthetized with ketamine/xylazine, and a catheter was placed into the cisterna magna. CSF samples were obtained for WBC count and determination of bacterial titers. ICP was measured. Finally, animals were perfused transcardially with ice-cold PBS, and brains were removed and either frozen immediately or fixed in formalin. Formalin-fixed brains were subsequently embedded in paraffin for immunohistochemistry.

Experimental groups in the mouse model
Acute model of pneumococcal meningitis. The following experimental groups were investigated: (a) WT mice injected intracisternally with 15 μl PBS (controls; C57BL/6, male, n = 8 and BALB/c, male, n = 6); (b) WT mice injected intracisternally with S. pneumoniae (C57BL/6, male, n = 12; C57BL/6, female, n = 20; and BALB/c, male, n = 10); (c) C3ar1−/− mice (male, genetic background C57BL/6; provided by Richard A. Wetsel, University of Texas Health Science Center, Houston, Texas, USA) injected intracisternally with S. pneumoniae (n = 12); (d) C3a/GFAP mice (male, genetic background C57BL/6) injected intracisternally with S. pneumoniae (n = 11); (e) C6−/− mice (female, genetic background C57BL/6) injected intracisternally with S. pneumoniae (n = 14); (f) C59a−/− mice (female, genetic background C57BL/6) injected intracisternally with S. pneumoniae (n = 11); (g) C5ar1−/− mice (male, genetic background BALB/c, obtained from The Jackson Laboratory) injected intracisternally with S. pneumoniae (n = 9); (h) WT mice injected intracisternically with S. pneumoniae and treated i.p. with either a neutralizing monoclonal antibody directed against murine C5 (1 mg per mouse; clone BBS.1, n = 7) (58, 59) or mouse IgG antibodies (1 mg per mouse, n = 12; Innovative Research); (i) WT mice injected intracisternally with S. pneumoniae and treated i.p. with a neutralizing monoclonal antibody directed against murine C3 (30 μg per mouse; clone BBS.1, n = 3), i.t. with a neutralizing monoclonal antibody directed against murine C5 (30 μg per mouse; clone BBS.1, n = 4), or i.t. mouse IgG antibodies (30 μg per mouse, n = 4); (j) WT mice injected intracisternally with S. pneumoniae and treated i.p. with 250 μg anti–GR-1 (granulocyte depletion antibody; n = 8) or mouse IgG antibodies (250 μg per mouse, n = 8); (k) WT mice injected intracisternally with S. pneumoniae and treated i.p. with a neutralizing monoclonal antibody directed against murine C5 (30 μg per mouse; clone BBS.1, n = 4), or i.t. mouse IgG antibodies (30 μg per mouse, n = 4); (l) WT mice injected intracisternically with S. pneumoniae and treated i.p. with 250 μg anti–GR-1 (granulocyte depletion antibody; n = 8) or mouse IgG antibodies (250 μg per mouse, n = 8).
For the determination of the BBB integrity, frozen mouse brain extracts the number of intracerebral hemorrhages were combined in a neuroscore. Brains were cut in a frontal plane into 10-mm thick sections. Beginning C5a and TCC were additionally evaluated by immunohistochemistry per digitized using a Zeiss Axiovert microscope (Carl Zeiss) connected to a size of 400 provides sufficient power (80%) when a risk genotype has a relative risk of more than 0.5 or a score value of 0.02 (Bonferroni corrected).

**Determination of cerebellar bacterial titer**

For determination of bacterial titer, the cerebellum was dissected and homogenized in 1 ml sterile PBS. Cerebellar homogenates were diluted serially, plated on blood agar plates, and cultured for 24 hours before CFUs were counted.

**Neuroscore**

For better comparison, the degree of breaching of the BBB integrity and the number of intracerebral hemorrhages were combined in a neuroscore. For the determination of the BBB integrity, frozen mouse brain extracts were examined for diffusion of albumin using ELISA as described previously (25). The score was 0, 1, or 2, if brain albumin was 0–35, 36–75, or 76–140 ng/μg, respectively. For more than 140 ng/μg of albumin, the score assigned was 3. For determination of intracerebral hemorrhage, mouse brains were cut in a frontal plane into 10-mm thick sections. Beginning from the anterior parts of the lateral ventricles, 9 serial sections were photographed with a digital camera at 0.3-mm intervals throughout the ventricle system. Hemorrhagic spots were counted, and the bleeding area was measured. A score of 0 indicates no cerebral bleedings, a score of 1 indicates up to 20 cerebral bleeding spots, a score of 2 indicates between 21 and 60 cerebral bleeding spots, and a score of 3 indicates more than 60 cerebral bleeding spots. The maximum neuroscore was 6 and indicated severe neuronal damage, whereas a score of 0 indicated no neuronal damage.

**Analysis of protein expression**

Expression of C5a, TCC, IL-6, CXCL1/KC, and CXCL2/MIP-2 was determined in mouse brain homogenates by ELISA according to the manufacturer’s instructions (C5a and TCC, USCN Life Science, Biozol; IL-6, CXCL2/MIP-2, and CXCL1/KC, R&D Systems). Expression profiles of C5a and TCC were additionally evaluated by immunohistochemistry performed on paraffin-embedded slides of mouse brain tissue as previously described (60). Briefly, after deparaffinization and steam bath antigen retrieval in citrate buffer, endogenous peroxidase was quenched with 7.5% hydrogen peroxide. Nonspecific binding was minimized by incubation in 10% normal goat serum. Slides were then incubated overnight at 4°C with a rat anti-mouse C5a or TCC antibody or the appropriate isotype control immunoglobulin. Specific labeling was detected with a biotin-conjugated rabbit anti-rat antibody and application of horseradish peroxidase–bound avidin/biotin from Vectastain ABC Kits, followed by development with 3,3′-diaminobenzidine (DAB) solution (both from Vector Laboratories). Counterstaining was performed using Mayer’s hematoxylin. Slides were digitized using a Zeiss Axiosvert microscope (Carl Zeiss) connected to a cooled Moticam 5000 video camera (Moticam).

**Statistical analysis – genetic analysis**

For evaluating the role of SNPs on outcome, assuming an overall event rate of 25% (n = 100 cases) to patients with favorable outcome (n = 300), a sample size of 400 provides sufficient power (80%) when a risk genotype has a relative risk of 3.0 or more, using a P value of 0.0029 (Bonferroni corrected). The Mann-Whitney U test was used to identify differences in baseline characteristics among groups with respect to continuous variables, and dichotomous variables were compared with use of the χ² test. These statistical tests were 2-tailed, and a P value of less than 0.05 was regarded as significant. Differences in genotype frequencies were analyzed with the χ² or Fishers’ exact tests by use of the programs R-statistics and PASW18. For the SNP analysis, we used a Bonferroni correction for multiple testing (17 SNPs; P < 0.0029). We calculated whether the genotype frequencies in the control groups concurred with the HWE by use of a χ² and exact test with 1 degree of freedom with a P value of less than 0.05 to indicate significance. SNPs deviating from the HWE were excluded.

The genotypic frequencies of patients with a favorable outcome was compared with those with an unfavorable outcome as defined by the GOS. Subgroup analyses were defined by ethnicity (mixed European descent), causative organism (S. pneumoniae), and a combination of these factors. We used a multivariate logistic regression analysis to calculate ORs and 95% CIs to assess the strength of the association among potential risk factors (including identified polymorphisms) and outcome.

**Statistics – animal experiments**

The principal statistical test was a 2-tailed unpaired Student’s t test (combined with an α-adjustment in case of multiple comparisons) or a logrank test (Mantel) for survival. Differences were considered significant at P < 0.05. Data are displayed as mean ± SD.

**Study approval**

The protocol used in this study was approved by the Academic Medical Center and all local participating hospitals (see Supplemental Methods). Written informed consent was obtained from all participating patients, or their legally authorized representatives, and controls. All animal experiments were approved by the animal ethic committee of the government of Upper Bavaria, Germany.

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