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Distinct Different Contributions of the Alternative and Classical Complement Activation Pathway for the Innate Host Response during Sepsis

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Complement activation represents a crucial innate defense mechanism to invading microorganisms, but there is an eminent lack of understanding of the separate contribution of the different complement activation pathways to the host response during sepsis. We therefore investigated different innate host immune responses during cecal ligation and puncture (CLP)-induced sepsis in mice lacking either the alternative (fD−/−) or classical (C1q−/−) complement activation pathway. Both knockout mice strains showed a significantly reduced survival and increased organ dysfunction when compared with control mice. Surprisingly, fD−/− mice demonstrated a compensated bacterial clearance capacity as control mice at 6 h post CLP, whereas C1q−/− mice were already overwhelmed by bacterial growth at this time point. Interestingly, at 24 h after CLP, fD−/− mice failed to clear bacteria in a way comparable to control mice. However, both knockout mice strains showed compromised C3 cleavage during sepsis. Investigating potential causes for this discrepancy, we were able to demonstrate that despite normal bacterial clearance capacity early during the onset of sepsis, fD−/− mice displayed increased inflammatory cytokine generation and neutrophil recruitment into lungs and blood when compared with both control- and C1q−/− mice, indicating a potential loss of control over these immune responses. Further in vitro experiments revealed a strongly increased Nf-κB activation capacity in isolated neutrophils from fD−/− mice, supporting this hypothesis. Our results provide evidence for the new concept that the alternative complement activation pathway exerts a distinctly different contribution to the innate host response during sepsis when compared with the classical pathway. The Journal of Immunology, 2011, 186: 3066–3075.

During acute inflammation, the complement system can be activated by at least three well-known pathways: the classical pathway, the lectin or mannose-binding lectin (MBL) pathway, and the alternative pathway (1). The classical pathway is activated, for example, by Ag–Ab complexes that react with activated C1q. The lectin pathway is initiated by either serum MBL or ficolins that recognize certain oligosaccharide moieties on microbial surfaces. The alternative pathway can be activated either by the presence of foreign surfaces such as LPSs or through C3b generated by spontaneous hydrolyses, the so-called “tick-over” (2). All three pathways merge at the level of C3, and activation of either pathway ultimately results in generation of the potent proinflammatory complement split products C3a and C5a as well as the terminal membrane attack complex (MAC) (3). Excessive generation of C5a during the onset of sepsis causes various harmful effects mediated by C5aR (1, 4, 5), and blockade of either C5a or C5aR leads to greatly improved survival of rodents in experimental sepsis (6, 7). C5a has been shown to alter innate immune functions, such as generation of inflammatory mediators as well as neutrophil functions, leading to a status of immune suppression (8). We recently demonstrated that C5a alters intracellular signaling pathways in neutrophils in vitro and during the onset of sepsis in vivo (9–12), offering an explanation for the above-mentioned suppression of innate immune functions.

Studies employing different complement knockout mice in a model of streptococcal pneumonia first suggested that the classical activation pathway appeared to be dominant for clearance of these bacteria (13). However, little is known about the contribution of the separate complement pathways during the onset phase of sepsis. Initial experimental infection studies with knockout mice lacking all three complement pathways demonstrated the importance of an intact complement activation system for successful survival, and results with C1q knockout mice showed involvement of the classical pathway (14, 15). Although both reports suggested importance of the alternative and lectin pathways, the individual contribution has not been dissected so far. We therefore conducted cecal ligation and puncture (CLP) studies with C1q−/− mice, lacking the classical pathway, and...
Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany) unless otherwise indicated.

CLP procedure in mice

Mice were anesthetized by i.p. injection of ketamine (Ketamin; DeltaSelect, Dreieich, Germany) and xylazine (Rompun; Bayer, Leverkusen, Germany). The cecum was exposed through a 2-cm abdominal midline incision and about two-thirds of the cecum was ligated. The ligated part of the cecum was punctured through and through with a 21-gauge needle. After repositioning the bowel, the abdomen was closed in layers, using a 5.0 surgical suture (Ethicon, Norderstedt, Germany). Mice were monitored for various signs of sickness every 6 h for 7 d.

CD4+/- mice were generated on the background of C57BL/6 mice as described previously by Bottro et al. (16). Factor D+/- mice were generated as described previously by Xu et al. (17), also on the background of C57BL/6 mice. Specific pathogen-free, C57BL/6 mice were used for control studies and obtained from Charles River, Sulzfeld, Germany. All animal studies were reviewed and approved by the local ethic committee of the state of Lower Saxony, Germany.

Collection of plasma samples in mice

After induction of CLP, animals were sacrificed at the indicated time points and blood was drawn using direct needle puncture of the heart. Blood samples were analyzed immediately after collection by automated veterinary hematology analyzer (PocH-100iV Diff, Sysmex, Leipzig, Germany).

For plasma collection, the samples were stored at 4˚C, centrifuged at 4700 x g for 10 min at 4˚C, and the plasma was collected and immediately snap-frozen in liquid nitrogen and stored at -80˚C until used for further analyses. For both plasma collection and whole blood analyses, EDTA or heparin was used as anticoagulant as warranted for the particular test.

C5a ELISA

The C5a concentration in various plasma samples was analyzed by the commercially available ELISA mouse complement component C5a DuoSet according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

Immunohistochemical staining for C3/C3b/C3c deposition in kidney sections

Immediately after sacrificing an animal, the organs were flushed with sterile physiological sodium chloride solution retrograde through the caudal caval vein, and the organs were harvested and snap-frozen in liquid nitrogen. The organs were embedded in Tissue-Tek OCT compound (Sakura Finetek, Heppenheim, Germany), and 6-μm tissue sections were prepared, air-dried, and then fixed in acetone for 10 min at -20˚C. Next, the tissue sections were washed with 0.01% TBST (Sigma-Aldrich Chemie), incubated with and then fixed in acetone for 10 min at 20˚C. After 30 min blocking with ChemMate Ab diluent (Dako, Hamburg, Germany), a polyclonal rabbit anti-human C3c Ab (Dako) in a dilution of 1:800 of the stock (0.5 mg/ml) in Tween 20 and TBS with 1% BSA (PAA Laboratories, Coelbe, Germany) was applied on the sections and incubated for 1 h at room temperature. The anti-C3c Ab used was specific for C3c, C3, and C3b with proven mouse cross-reactivity according to the manufacturer’s manual. Thereafter, the tissue sections were washed for 2 min with TBST before incubation with undiluted EnVision+/HRP, Rb (Dako) for 30 min at room temperature. After washing with TBST the sections were stained with diaminobenzidine solution (0.05% diaminobenzidine/0.01% H2O2 in TBST) (Dako). Counterstaining was achieved with Mayer’s hemalum (Dako) for 10 min. Tissue sections were fixed and cover slides were mounted with Vitro-Cld (Langenbrinck, Emmelndorf, Germany). Staining was documented using light microscopy (Axiophot; Zeiss, Jena, Germany) and digital imaging (Spot Advanced software; Visitron Systems, Puchheim, Germany).

Quantitation of organ dysfunction during sepsis in vivo

To determine organ dysfunction of various organs in vivo, plasma samples were collected from the various groups of mice at different time points after CLP and analyzed for urea, bilirubin, glutamate oxaloacetate transaminase/aspartate transaminase (GOT/AST), and lactate dehydrogenase (LDH) using an automated clinical chemistry analyzer (Fujif Dri-Chem 3500i; Sysmex).

Bacterial load in organs from CLP animals

Six hours and 24 h after CLP blood, lung, liver, and kidneys were isolated from wild-type, D/-, and C1q/- mice in a sterile manner. Then, the organs were washed in a 70% ethanol bath for 10 s to avoid surface bacterial contamination. Each organ was manually homogenized in 3 ml sterile 0.9% NaCl. Then, 2 ml enriched brain-heart infusion was added. Samples were plated on sheep blood agar or anaerobic blood agar and incubated for 48 h at 37˚C under aerobic and anaerobic conditions. CFU were then determined in all plates and multiplied by the dilution factor. Data are presented as CFU per 50 μl nondiluted blood or CFU per 200 mg nondiluted tissue homogenate.

Quantitation of phagocytosis in whole blood cells

To determine the ability of blood granulocytes and monocytes to conduct phagocytosis, flow cytometric-based assay was used according to the manufacturer’s instructions (Phagotest; Orpegen Pharma, Heidelberg, Germany). Mouse whole blood samples were incubated with FITC-labeled Escherichia coli bacteria (1.7 x 108 bacteria/ml) for 20 min in a 37˚C warm water bath. Leukocyte surface-bound bacteria were neutralized using quenching solution. Cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). In a forward/side scatter dot plot, gates were set on granulocytes and monocytes to analyze each population with regard to their mean fluorescence intensity.

Quantitation of myeloperoxidase in the lung

Immediately after sacrificing the animals, organs were flushed with sterile physiological sodium chloride solution retrograde through the caudal caval vein, and the organs were harvested and snap-frozen in liquid nitrogen. Tissue homogenates were prepared according to the manufacturer’s instructions and the myeloperoxidase (MPO) concentration was analyzed by a commercially available ELISA (Hycult Biotechnology, Uden, The Netherlands). The protein content of the homogenates was determined with Bradford’s method.

Quantitation of MPO in plasma samples

Plasma samples were collected as outlined above. The MPO concentration in various plasma samples was analyzed by a commercially available ELISA according to the manufacturer’s instructions (Hycult Biotechnology).

Determination of histological organ damage during sepsis in vivo

To visualize organ damage on the histological level, we conducted CLP studies in the various mouse strains and isolated fresh organs at 0, 6, and 24 h after CLP immediately after sacrificing the animals. Organs were fixed for 24 h in 5% formaldehyde, embedded in paraffin (Leica TP 1020 tissue processor, Leica EG1160 embedding center) and sectioned at a thickness of 3 μm, placed on glass slides, and stained with H&E according to the following protocol: The tissue sections were deparaffinized and rehydrated with xylene and decreasing graded ethanol (100, 100, 96, 80, 60, 50%), dyed for 2 min with Mayer’s hemalum, rinsed for 10 min in water, counterstained for 7 min with eosin, and rinsed with water again. All reagents used for histology were purchased from Roth (Karlsruhe, Germany). Staining was documented using light microscopy (AxioVision; Zeiss).

Quantitation of IL-6, MCP-1, IFN-γ, and TNF-α

Plasma samples were collected as outlined above. For quantification of various mediators, a commercially available flow cytometric bead assay (CBA) was performed according to the manufacturer’s instructions (mouse inflammation kit; BD Biosciences, Heidelberg, Germany).

Isolation, culture, and activation of peripheral murine leukocytes

Peripheral leukocytes from whole blood of all mouse strains were separated. Whole blood was drawn from 24 untreated mice of each strain as described above. In the first step platelet-rich plasma was demounted after centrifugation (15 min, 80 x g, room temperature plus 12 min, 240 x g, room temperature). Cells were purified from erythrocytes using ammonium chloride lysis buffer (0.155 M NH4Cl, 0.01 M KHCO3, and 0.1233 M
EDTA (pH 7.27)); and the remaining leukocytes were cultured for 2 or 24 h (RPMI 1640 medium containing 10% mouse serum and 1% Gluta-
Max, 37°C, 5% CO2). Afterwards, the leukocytes were left untreated or were stimulated with 200 ng/mL LPS for 30 min. After 24 h incubation the cells were lysed with NETN buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5% Nonidet P-40, 0.5 mM PMSF, proteasine inhibitor mixture 1:100) with sonication following. The pro-
tein content of the homogenates was determined with Bradford’s method.

NF-κB generation in isolated mouse leukocytes: avidin-biotin complex DNA assay and Western blot

The avidin-biotin complex DNA assay is based on immobilization of protein-DNA complexes via binding of a biotinylated oligonucleotide to a streptavidin matrix. Biotinylated oligos were used from Biomers.net (Ulm, Germany) and were composed of the following sequences: NF-κB consensus oligonucleotide, sense, 5′-AGT TGA GGG GAC TTT CCC TCC GAG C-3′ and antisense, 5′-GCC TGG GAA AGT CCC CTC AAC T-3′. A total of 200 μl leukocyte whole-cell extract (same protein content) was incubated with 200 μl buffer H (50 mM KCl, 20 mM HEPES [pH 7.8], 20% glycerol, 1 mM DTT, 0.1% Nonidet P-40), 2 μl biotinylated oligo, 10 μl herring sperm DNA, and 5 μl 2 M KCl were boiled for 5 min at 37°C and stored on ice for 1 h. After addition of 41 μl equilibrated streptavidin agarose beads (Novagen, Madison, WI), incubation was continued for 30 min at 4°C on a rotator. Beads were washed repeatedly with buffer H and boiled in Laemmli sample buffer, and proteins were separated via SDS-PAGE under nonreducing conditions. NF-κB (65 kDa) was detected by Western blot (anti-NF-κB p65 Ab P056 Ab C22B4; Cell Signaling Technology, Danvers, MA) via chemiluminescence detection (Immobilon Western chemiluminescent HRP substrate; Millipore, Billerica, MA). Gray scale value density of detected results was analyzed by Aida Image Analyzer v.3.52 (Raytest, Straubenhardt, Germany). To demonstrate the equal protein load in SDS-PAGE, β-actin (45 kDa) was detected in Western blot, too (β-actin [13E5]; Cell Signaling Technology, Danvers, MA). Cell supernatants were also obtained after 2 h incubation and analyzed for cytokine generation using CBA assays as described above.

Statistical analysis

All values were expressed as the mean ± SEM. Significance was assigned where p < 0.05. Significances of normal distributed data were identified using one-way ANOVA followed by post hoc comparisons using Bonfer-
noni–Holm correction. Statistical analyses of data that did not follow normal distribution were conducted by the nonparametric Kruskal–Wallis test followed by a Dunn’s post test. The software used was GraphPad Prism 5.0 (GraphPad Software).

Results

Outcome in experimental sepsis CLP study in wild-type, fD−/−, and C1q−/− mice

To investigate the effect of impaired activation of the classical complement activation pathway in C1q−/− mice and the impaired activation in fD−/− mice on outcome in experimental sepsis, we conducted CLP experiments in these mice. Mice were followed up for 7 d and monitored every 6 h for various signs of sickness. C1q−/− mice started to die very rapidly after 24 h and after 55 h none of the animals survived (Fig. 1). Although fD−/− mice survived slightly longer, the mortality also reached 100% after approximately 4 d. Wild-type mice displayed a significantly higher survival rate at ~30%. These results demonstrated an impaired survival capacity of C1q−/− and fD−/− mice when compared with the wild-type controls, suggesting an important role for the classical and the alternative pathway for successful host response during sepsis. We therefore tried to investigate whether the expected underlying lack of overall complement activation and therefore a lack of bacterial clearance capacity would be a sufficient explanation for these findings.

C5a generation in plasma from wild-type, fD−/−, and C1q−/− mice

To underline the comparability of the used mouse strains with respect to their ability to generate one known key player, C5a, we measured C5a concentrations in plasma by ELISA. There were no detectable differences in baseline C5a generation in plasma of untreated knockout or control mice (Fig. 2A). Control mice as well as C1q−/− mice displayed an expected increase of C5a levels at 3 h after CLP, which was most pronounced in C1q−/− mice. The latter also displayed a statistically significant initial drop at 1.5 h after CLP when compared with control mice (Fig. 2B). Even though fD−/− mice showed some decrease in C5a levels at 6 h, these changes were not statistically different from control levels. These results demonstrated that fD−/− mice were capable of generating equal baseline C5a levels when compared with control and C1q−/− mice, but they displayed a lowered and timely delayed increase in C5a generation after the onset of sepsis.

Immunohistochemical staining for C3/C3b/C3c deposition during sepsis in kidney sections from wild-type, C1q−/−, and fD−/− mice

To determine the contribution of the classical and the alternative pathway for C3 activation, we detected C3/C3b/C3c deposition in the kidney of wild-type, C1q−/−, and fD−/− mice 3 h after CLP.
significantly worsened organ dysfunction in these mice. LDH was compared with wild-type and fD mice. Similar observations were made in the renal cortex section with mild C3c deposition in the glomeruli in wild-type mice that was strongly increased 3 h after CLP, whereas no C3/C3b/C3c deposition was detected in both complement knockout mice strains (data not shown). These data indicated a crucial contribution of both the classical and the alternative pathway to overall C3 cleavage and therefore activation of the complement system in sepsis.

Effects of fD and C1q knockout on organ dysfunction during CLP-induced sepsis

To establish whether the lack of factor D or C1q could be specifically linked to organ dysfunction during experimental sepsis, we analyzed organ-specific indicators in the plasma of septic mice at 6 and 24 h after induction of CLP (Fig. 4A–D). All groups, C1q−/−, fD−/−, and wild-type control mice, demonstrated elevated levels of the following parameters when compared with their corresponding control values at 0 h CLP: urea, indicating a reduced filtrating function of the kidneys (Fig. 4A); GOT/AST, indicating liver cell damage (Fig. 4C); bilirubin, indicating a reduced liver capacity to convert hemoglobin and/or excrete bilirubin (Fig. 4B); and LDH as an indicator for tissue and organ damage (Fig. 4D). Wild-type, fD−/−, and C1q−/− mice displayed significantly increased values of urea, GOT/AST, and LDH at 24 h after CLP when compared with control mice (Fig. 4A, 4C, 4D), indicating significantly worsened organ dysfunction in these mice. LDH was significantly elevated in C1q−/− mice 24 h after CLP when compared with wild-type and fD−/− mice. In case of bilirubin, only fD−/− and C1q−/− mice showed significantly increased levels 24 h after CLP, which could not be observed in wild-type mice. In summary, we observed a tendency toward more pronounced organ dysfunction in fD−/− and C1q−/− mice when compared with control mice, with these effects being most prominent in fD−/− mice. No significant increases during the onset of sepsis were found in any of the three groups for alkaline phosphatase and plasma albumin (data not shown).

Bacterial load in blood, lung, liver, and kidney

Bacterial clearance represents one major endpoint for innate host immune response to infection that is thought to be greatly influenced by intact complement activation and formation of the terminal complement activation products. We therefore sought to determine the capacity of alternative and classical complement pathway knockout mice to clear bacteria during the onset of sepsis in comparison with untreated control mice. Given the above-described lowered survival and increased organ dysfunction in fD−/− and C1q−/− mice, we expected to find increased bacterial loads in both knockout strains. C1q−/− mice demonstrated significantly elevated aerobic bacterial loads in lungs, liver, and blood and a strong but not significant similar tendency in kidney 6 h after CLP when compared with control mice (Fig. 5A–D). Surprisingly, fD−/− mice did not demonstrate such increases in bacterial organ loads and demonstrated an equal capability of clearing bacteria when compared with control mice. However, 24 h after CLP this situation changed and aerobic bacterial loads showed an increase compared with the other strains, which was statistically significant for liver samples and present as a strong tendency in blood and kidney samples (Fig. 5E–H). Anaerobic bacterial loads displayed a higher level of variance between different study animals after 6 and 24 h, but showed similar tendencies when compared with aerobic results (data not shown).

Effects of C1q and fD knockout on phagocytosis activity in granulocytes and monocytes during sepsis

In light of the above detailed findings of increased bacterial loads in C1q−/− mice, we sought to investigate whether the phagocytosis activity in granulocytes and monocytes would also be affected by impaired activation of the alternative or classical complement cascade. Whole blood samples were collected at 0 and 6 h after CLP from wild-type, fD−/−, and C1q−/− mice and then stimulated with opsonized FITC-conjugated E. coli bacteria (1.7 × 10⁷/ml) for 20 min. Phagocytosis activity was then measured using a flow cytometer. Granulocytes from wild-type mice demonstrated a comparatively high baseline ability to phagocytose bacteria, which significantly decreased 6 h after CLP (Fig. 6A). Both fD−/− and C1q−/− mice showed only marginal ability to phagocytose bacteria under untreated conditions. However, there was a somewhat inducible potential for phagocytosis in C1q−/− mice 6 h after CLP, which was absent in fD−/− mice (Fig. 6A). Similar results were obtained in monocytes (Fig. 6B). These findings could explain in part the reduced bacterial clearance capacity during sepsis in C1q−/− mice. However, despite the detected low phagocytosis capacity in fD−/− mice, the latter group was capable of clearing bacteria similarly well when compared with control mice.

MPO content in the lung and plasma during sepsis in wild-type, fD−/−, and C1q−/− mice

To better understand and interpret the findings in our phagocytosis and bacterial clearance experiments, we evaluated the invasion of neutrophils into the lung during the onset of sepsis by measuring the amount of MPO with ELISA technique in homogenized lung tissue samples from wild-type, fD−/−, and C1q−/− mice before (CLP 0 h) and 6 h after CLP. The concentration of MPO was normalized to 100 µg protein. In all three groups (wild-type, fD−/−, and C1q−/− mice) the MPO concentration was significantly lower in septic fD−/− mice than in the other groups, indicating a reduced bacterial clearance capacity in fD−/− mice. However, the C1q−/− mice showed a similar tendency in the lung and plasma (data not shown).

**FIGURE 3.** Immunohistochemical staining for C3c deposition in kidney sections during sepsis from wild-type, C1q−/−, and fD−/− mice. Compared are C3c staining results from kidney sections from wild-type (top panel), C1q−/− (middle panel), and fD−/− mice at 0 h (left panel) and 3 h after CLP (right panel). Results are representative of three to six staining experiments from two independent experiments per condition. Original magnification ×200. wt, wild-type.
elevated at 6 h after CLP when compared with the corresponding 0 h control groups (Fig. 7A). In fD−/− mice the total MPO concentrations as well as the increase in MPO content were significantly higher when compared with C1q−/− or wild-type mice at 6 h after CLP (Fig. 7A, 7C). To assess the amount of MPO secreted or released from neutrophils in the plasma during the onset of sepsis, we measured the MPO concentration with an ELISA in plasma samples from wild-type, fD−/−, and C1q−/− mice at 0 and 3 h after CLP. In all three groups (wild-type, fD−/−, and C1q−/− mice), significantly higher MPO concentration were measured 3 h after CLP when compared with the corresponding 0 h control groups (Fig. 7B). Again, the total MPO concentrations in plasma, as well as the increase in MPO in fD−/− mice at 3 h after CLP, were significantly higher when compared with wild-type or C1q−/− mice (Fig. 7D). These results indicated that fD−/− mice could potentially compensate their low phagocytosis capacity by a significantly increased neutrophil recruitment to reach an overall normal bacterial clearance capacity when compared with healthy control animals.

**Effects of C1q and fD knockout on structural organ changes during CLP-induced sepsis**

In light of the above findings, demonstrating rapid mortality in fD−/− and C1q−/− mice during CLP-induced sepsis, we were able to demonstrate significantly increased organ dysfunction in both knockout strains when compared with control mice 24 h after CLP. We therefore conducted additional staining experiments for organ sections from septic animals of the above-mentioned groups of mice to visualize the potential extent of structural organ changes in livers (not shown) and lungs from septic animals (Fig. 8). In all groups, signs of inflammation were observed 6 and 24 h post CLP. However, lungs from fD−/− and C1q−/− mice demonstrated more signs of severe structural organ changes, such as alveolar swelling and infiltration of mononuclear cells, when compared with organ sections from wild-type mice, which displayed such changes to a lesser extent. Structural organ changes appeared to be most pronounced in fD−/− mice. These findings were in line with the results of the organ dysfunction parameters and suggested an overall deleterious effect of fD−/− and C1q−/− knockout during the onset of sepsis.

**Cytokine generation during sepsis in wild-type, fD−/−, and C1q−/− mice**

To gain more information about the cause of death in fD−/− mice, which apparently were capable of undisturbed bacterial clearance, we investigated differences in cytokine patterns in whole blood during CLP-induced sepsis 6 and 24 h after CLP in wild-type, fD−/−, and C1q−/− mice (Fig. 9). Because production of inflammatory mediators reflects one arm of the innate host response, which is thought to be directly dependent on the concentration of pathogen-associated molecular patterns (PAMPs) and therefore also on the bacterial load, we predicted that we would find the highest levels of such mediators in C1q−/− mice. As depicted in Fig. 9, in all three groups the serum levels for TNF-α (Fig. 9B), IL-6 (Fig. 9A), MCP-1 (Fig. 9D), and IFN-γ (Fig. 9C) were significantly increased 24 h after CLP when compared with corresponding 0 h values. It is known that wild-type mice typically already demonstrate very high cytokine levels in this sepsis model. However, in both knockout strains, IL-6, TNF-α, and IFN-γ generation was significantly higher 24 h after CLP when compared with wild-type controls (Fig. 9A–C). In fD−/− mice, levels of IL-6 and TNF-α even demonstrated a tendency toward higher levels at 24 h after CLP when compared with C1q−/− mice, which did not reach statistical significance (Fig. 9A, 9B). With respect to the earlier described normal bacterial clearance capacity of fD−/− mice, these results suggested that these mice potentially suffered from a loss of control over the host cytokine response to bacterial challenge or that these mice were capable of producing significantly increased cytokine amounts through the established increased neutrophil recruitment alone.

**NF-κB generation and cytokine generation in isolated mouse leukocytes**

Based on the above-depicted findings, we hypothesized that factor D may exert a controlling function for cytokine generation and investigated the ability of fD−/− mice to activate NF-κB in isolated leukocytes upon stimulation with bacterial LPS as a prototype

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**FIGURE 4.** A–D, Analysis of various blood and plasma parameters indicative of organ dysfunction during sepsis in wild-type, fD−/−, and C1q−/− mice. Automated plasma parameter analysis for urea, bilirubin, GOT/AST, and LDH in plasma samples from septic mice at different time points after CLP. *p < 0.05, **p < 0.01, ***p < 0.001 for statistical significance to 0 h value of the groups. *p < 0.05, ##p < 0.01, ###p < 0.0001 for statistical significance between different groups. Data are representative of 10–12 animals per group and are depicted as mean ± SEM. wt, wild-type.
PAMP. We found that NF-κB activation, as detected in leukocytes pooled from 24 mice per experiment upon stimulation with LPS, reached higher levels in fD/2 mice when compared with wild-type and C1q/2 mice (Fig. 10A), which became even more evident after grayscale value density analysis (Fig. 10B).

To investigate the significance of this finding for mediator generation in isolated mouse leukocytes ex vivo, supernatants of leukocytes pooled from 24 mice per strain were obtained 2 h after stimulation with LPS and analyzed for cytokine generation using CBA assay. The results in Fig. 10C–F demonstrated that, corresponding to the findings during sepsis in whole blood, leukocytes from fD/2 mice generated higher levels of IL-6, TNF-α, MCP-1, and IFN-γ when compared with neutrophils from C1q/2 and control mice. These results suggested a potential controlling function of factor D for NF-κB–dependent cytokine generation in mouse leukocytes.

**Discussion**

It is well known that sufficient activation of the complement system during infection is essential for a successful immune response and defense. However, the individual contributions of the separate complement activation pathways for known changes in host defense and innate immune functions during sepsis have not yet been investigated in detail. Knockout mice lacking all three complement activation pathways demonstrated extreme susceptibility to infection caused by bacterial challenge, and results with C1q/2 mice demonstrated involvement of the classical pathway (14). Similar results were previously found using these two knockout strains in the CLP sepsis model (15). Both reports suggested...
importance of the alternative pathway, but the separate contribution could not be determined at that time. To investigate the effects of impaired alternative complement activation in comparison with defective classical complement activation on outcome and impaired innate immune responses during experimental sepsis, we conducted CLP studies using C1q\(-/-\) and Fd\/-/ mice, which have been shown to lack specifically the alternative pathway (17). In line with previous studies (15), we found C1q\(-/-\) mice to be highly susceptible, with 100% mortality very early during CLP-induced sepsis. Factor D\/-/ mice also displayed 100% mortality but survived 2 d longer in the mean values (Fig. 1). Interestingly, Fd\/-/ mice displayed normal baseline C5a levels when compared with control mice (Fig. 2A). The same was true for C1q\/-/ mice.

It has been previously reported that C57BL/6 mice, which served at least partially as background also for both knockout, appear to have some defect in the classical C5 convertase, leading to compromised classical C5 activation (18). Our results in this study suggest that both knockout strains employed in our studies did not suffer from an inability to generate C5a. However, it is known now that C5a can be generated in the absence of the classical or alternative convertase (19). Surprisingly, Fd\/-/ mice displayed a different characteristic in C5a levels in the time course of sepsis compared with wild-type and C1q\/-/ mice (Fig. 2B), and they demonstrated no significant changes over time. Additionally, C1q\/-/ mice exhibited a decrease after 1.5 h after CLP, which was followed by a significantly higher increase after 3 h compared with Fd\/-/ and wild-type mice, which could be a possible cause for the rapid onset of mortality. Besides the possibility of local additional generation independent of upstream complement activation, there are various other factors influencing C5a levels in plasma. One of them is degradation, which is dependent on availability and activation status of the degrading C5 processing enzymes. Another, even more important factor is the existing large C5a sink in form of an abundant number of C5a receptors such as C5aR and C5L2 (20) on almost all tissues and especially on blood neutrophils. C5aR is dramatically upregulated during sepsis in C5aR and C5L2 (20) on almost all tissues and especially on blood neutrophils. C5aR is dramatically upregulated during sepsis in various tissues (7). Because all of these factors display strong dynamics during sepsis, we must conclude that measured C5a levels in plasma may not accurately reflect the extent of the activation of the complement system.

Experiments with C3\/-/ mice have demonstrated before that these animals are highly susceptible to a broad range of infectious insults (3, 21–23), with these mice being incapable of generating the important opsonin C3b and initiating the formation of the MAC. In contrast, there is accumulating evidence that excessive production of the potent proinflammatory complement split product C5a exerts harmful effects on innate immune functions and worsens outcome during experimental sepsis (1, 4–7). These findings are in line with our findings in factor D knockout mice with respect to the outcome studies. The increased susceptibility in both C1q and factor D knockout mice could be explained by the strongly reduced activation of C3 and the MAC, as suggested by our staining experiments for C3c deposition in the kidneys (Fig. 3).
We showed that C1q$^{-/-}$ mice are not able to clear bacteria effectively during the early onset of sepsis, whereas fD$^{-/-}$ mice were fully capable of clearing bacteria 6 h after induction of sepsis when compared with C1q$^{-/-}$ and wild-type mice (Fig. 5A–D). Our findings therefore confirm the important role of the classical pathway for survival during sepsis by being mainly responsible for...
intact clearance of bacterial challenges early during sepsis. Interestingly, we found that the bacterial clearance at 24 h after CLP displayed a different situation when compared with the 6 h values: ID<sup>−/−</sup> mice now presented with a significantly increased bacterial load in liver when compared with wild-type and C1q<sup>−/−</sup> mice and a similar, but not statistically significant, tendency for kidney and whole blood (Fig. 5E–H). These findings add a new aspect since the importance of the separate pathways for bacterial clearance appears to be time-dependent. The classical pathway is apparently very important for clearing bacteria in the early development of sepsis, whereas the alternative pathway may play a more important role for the later phase of development.

A recent report suggested an important protective role of properdin, a cofactor of the alternative complement system, for outcome during experimental sepsis (24). These findings are in line with our findings in ID<sup>−/−</sup> mice with respect to the outcome results, demonstrating an important role of the alternative pathway for survival during experimental sepsis. A critical role for the alternative pathway has been also identified before for survival of mice infected with Pseudomonas aeruginosa in a murine model of pneumonia (22). In vitro studies with serum from ID<sup>−/−</sup> mice demonstrated that the alternative pathway strongly contributed to the deposition of C3 on the phosphocholine-rich surface in kidneys. The authors therefore suggested that this pathway critically contributed to overall complement activation and was important for opsonization and clearance of bacteria (17). Even though our observations for C3b staining in kidneys emphasize that ID<sup>−/−</sup> mice also demonstrate a compromised ability for C3 cleavage during sepsis, our new findings demonstrating a normal bacterial clearance of these knockout mice in early CLP-induced sepsis when compared with control mice suggest an additional role of this pathway and of factor D during sepsis other than only contributing to overall complement activation.

In our studies, both knockout strains showed a strongly reduced baseline phagocytosis capacity when compared with control mice (Fig. 6), which could also be explained by functional defects in neutrophils of these knockout strains, but ID<sup>−/−</sup> mice depicted significantly higher MPO levels in lung and whole blood (Fig. 7). We therefore hypothesize that an altered and somewhat less controlled neutrophil recruitment in ID<sup>−/−</sup> mice may compensate for their reduced phagocytosis capability, leading to an overall normal bacterial clearance in comparison with control mice at least early during the onset phase of sepsis. Additional histological experiments showed pronounced mononuclear cell infiltrations in lungs and liver of ID<sup>−/−</sup> mice in contrast to wild-type and C1q<sup>−/−</sup> mice (Fig. 8), supporting this hypothesis.

We investigated cytokine generation in these mice (Fig. 9) and found that ID<sup>−/−</sup> mice displayed extremely high levels of IL-6, TNF-α, MCP-1, and IFN-γ when compared with the cytokine storm-like values already present in control mice during sepsis. These findings further suggested some kind of loss of control over PAMP-induced inflammatory mediator generation during sepsis and could explain a worsened outcome in these animals, as it is well known that inflammatory mediators such as IL-6 and TNF-α exert harmful effects per se, leading also to death in mice when administered in higher doses. Because cytokine changes are usually delayed several hours from maximum stimulus to maximum observed phenotype (stimulation of isolated cells), it may well be possible that, depending on the cytokine, the depicted 24 h levels in whole blood might reflect the bacterial load differences present earlier, such as 6 h after CLP. To further elucidate the hypothesis that ID<sup>−/−</sup> mice generate higher levels of inflammatory mediators even though similar stimuli may be present, we investigated the ability of neutrophils from ID<sup>−/−</sup> mice to induce NF-κB activation upon infectious stimuli (Fig. 10) and found this to be strongly enhanced. NF-κB activation is well known to promote cytokine generation (25) as well as mechanisms leading to neutrophil recruitment (26). We therefore suggest this mechanism to be at least partially responsible for the observed increases in inflammatory mediator generation and increased MPO levels in lung and whole blood. Note that based on the knockout of a soluble serum factor such as factor D, it may not directly be expected that cell-derived effects can be observed. Based on the available data, we can currently only speculate that factor D may be involved in controlling NF-κB activation in blood neutrophils and that knockout of this factor may lead to increased cell signaling via NF-κB. Further experiments are warranted to define the underlying mechanisms in more detail.

Interestingly, in models of chronic-type noninfectious inflammatory diseases, such as asthma or autoimmune nephropathy, inactivation of the alternative pathway showed beneficial effects, partially by lowering the inflammatory response (2, 27, 28). In these models, the activation of the alternative pathway was suggested to act as an amplification loop triggering the hyperactive inflammatory response and thereby contributing negatively to the progression of the disease, whereas in models of bacterial infection an intact activation of the complement system seemed to be essential for successful and early control of the infection as discussed above. It is therefore likely that the alternative pathway plays a different role in aseptic inflammatory responses when compared with immune responses during septic inflammation. In a model of gastrointestinal ischemia and reperfusion injury, the third pathway, the MBL-activated pathway, has been demonstrated to be mainly responsible for gastrointestinal injury but not for development of lung injury (29). In this model, the alternative pathway has also been suggested to act as an amplifier for complement activation with critical initial activation of the lectin and/or classical complement pathway (30). Similarly, myocardial ischemia/reperfusion injury was dependent on MBL-dependent pathway activation, but not on the activation of the classical pathway (31, 32) as suggested by earlier reports (33). Evidence exists also for a key role of the lectin pathway in infection, as MBL A/C<sup>−/−</sup> mice have been shown to be highly susceptible to Staphylococcus aureus and to postbacterial infection with P. aeruginosa (34, 35). Taken together, throughout the literature, one proposed main function of the alternative pathway has been the amplification of complement activation after initial activation of the classical or MBL pathway (30, 36, 37). However, our recent study suggests a potentially new controlling and time-dependent function of this pathway during experimental sepsis in mice. In a recent study from our group, we were able to demonstrate a similar direct regulatory function on the innate immune response of the host for the PI3K pathway (9). In that study, PI3K-deficient animals also displayed strongly increased inflammatory responses and reduced phagocytosis function when compared with control animals during sepsis, leading to worsened outcome.

We conclude from our findings an important and previously unrecognized controlling function of the alternative complement activation pathway, and particularly of complement factor D, for various innate immune responses during experimental sepsis. We further provide evidence for our hypothesis that the amplifying function of the alternative complement pathway for overall complement activation as suggested in other models of inflammation appears not to be a major mechanism in experimental sepsis. Our report therefore suggests distinct different functions of these two complement activation pathways for host innate immune responses in experimental sepsis.
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References