Antibody-Based Immunotherapy To Treat and Prevent Infection with Hypervirulent Klebsiella pneumoniae

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ABSTRACT Hypervirulent Klebsiella pneumoniae (hvKp) strains are predicted to become a major threat in Asia if antibiotic resistance continues to spread. Anticapsular antibodies (Abs) were developed because disseminated infections caused by hvKp are associated with significant morbidity and mortality, even with antibiotic-sensitive strains. K1-serotype polysaccharide capsules (K1-CPS) are expressed by the majority of hvKp strains. In this study, K1-CPS-specific IgG Abs were generated by conjugation of K1-CPS to immunogenic anthrax protective antigen (PA) protein. Opsonophagocytic efficacy was measured in vitro and in vivo by intravital microscopy in murine livers. In vivo protection was tested in murine models, including a novel model for dissemination in hvKp-colonized mice. Protective efficacy of monoclonal antibodies (MAbs) 4C5 (IgG1) and 19A10 (IgG3) was demonstrated both in murine sepsis and pulmonary infection. In hvKp-colonized mice, MAb treatment significantly decreased dissemination of hvKp from the gut to mesenteric lymph nodes and organs. Intravital microscopy confirmed efficient opsonophagocytosis and clearance of bacteria from the liver. In vitro studies demonstrate that MAbs work predominantly by promoting FcR-mediated phagocytosis but also indicate that MAbs enhance the release of neutrophil extracellular traps (NETs). In anticipation of increasing antibiotic resistance, we propose further development of these and other Klebsiella-specific MAbs for therapeutic use.

KEYWORDS Klebsiella pneumoniae, hypervirulence, adjuvant therapy, monoclonal antibodies, colonization, opsonophagocytosis, intestinal colonization

Multidrug-resistant Klebsiella pneumoniae strains have kept the medical community on high alert, as they have been spreading worldwide. The most successful clonal type in the United States is the ST258 clone (1), although other clones have been described as well (2). Less noticed but also concerning is the emergence of hypervirulent K. pneumoniae (hvKp) strains. These K. pneumoniae strains are phenotypically easily identified in the laboratory because they express a hypermucoid polysaccharide capsule, which traditional serotyping classified as a K1 serotype. These hvKp strains are much more virulent than the ST258 strain and kill mice in a few days (3). They cause community-acquired infections that rapidly progress to invasive disease in patients without major comorbidities (3). These strains have been reported worldwide (4, 5), but the majority of cases are in Asia, where hvKp is the leading cause of liver abscesses in Taiwan (6), Singapore (7), Hong Kong (8), and South Korea (9). In fact, in Taiwan, the annual incidence of pyogenic liver abscess has increased between 1996 and 2004 from 11 to 17 cases per 100,000 persons (10). A significant percentage of hvKp infections

disseminate to the eye or meninges (11), commonly resulting in irreversible damage and mortality rates ranging from 3% to 42% (3). Epidemiological studies demonstrated that healthy adults carry virulent strains in their gastrointestinal tracts, and multilocus sequence typing (MLST) confirms a link between colonization and invasive infections in patients (12).

Fortunately and in contrast to ST258 strains (13), hvKp strains exhibit less diversity of their capsular polysaccharide (CPS). The K1 serotype is the prevailing CPS and is present in up to 81% of hvKp strains, followed by K2 and non-K1/K2 serotypes, respectively (3, 11). Most K1 serotype strains, but not all, are assigned to the ST23 clonal group by MLST (14). Until recently, hvKp strains were sensitive to standard antibiotics, so, if recognized early, infections caused by hvKp could be successfully treated despite their invasive nature. In vitro experiments demonstrated successful transfer of a KPC-bearing plasmid to an hvKp strain (15) and raised worries that blaKPC-2 and blaKPC-3 could emerge in an hvKp strain and cause infections in the community. In support of these concerns are reported rising antibiotic resistance in hvKp strains in China (16). Indeed, in 2014 and 2015 the first carbapenem-resistant hvKp strains were isolated from patients in China (17, 18).

Given the looming threat of emerging multidrug-resistant hvKp strains, we sought to develop novel therapeutic antibodies (Abs) targeting the CPS of these hvKp strains.

We present investigations with two monoclonal antibodies (MAbs) that promote phagocytosis by Kupffer cells in the liver and exhibit protective efficacy in three murine models, including one novel model that examines the effect of MAb treatment on dissemination in colonized mice.

**RESULTS**

**Immunization with PA-conjugated K1-CPS.** Vaccination with K1-CPS conjugated to protective antigen (PA) enhanced immunogenicity and yielded 6 IgG-producing hybridomas (3 IgG1, 2 IgG3, and 1 IgG2a). The two selected hybridomas, 4C5 (IgG1) and 19A10 (IgG3), excreted high-affinity K1-CPS-specific MAbs. Coincubation with either MAb induced agglutination and quelling of 3 distinct K1 K. pneumoniae strains (Fig. 1A), whereas no agglutination was observed with 47 control non-K1 K. pneumoniae strains (13 and data not shown). Variable region (VR) sequence analysis established that the heavy chain (V_H) of 19A10 is identical to that of the IGHV2-9*02 family, whereas the V_H of 4C5 is 95.79% identical (8 mutated residues) to the sequence of IGHV1S41*01 family members (Table 1). The light chain (V_L) of 19A10 is identical to that of IGKV1-117*01, and that of 4C5 is 99.64% identical (1 base mutated) to the IGKV6-25*01 germ line kappa sequence. Enzyme-linked immunosorbent assays (ELISAs) employing isotype-specific secondary Abs demonstrated simultaneous binding to purified CPS, consistent with nonoverlapping epitopes (data not shown).

**4C5 and 19A10 promote complement deposition and NETosis.** Although C3 complement (C3c) deposition on the capsule in the presence of binding of MAbs 4C5 and 19A10 was significantly increased by 44% and 17% compared to the phosphate-buffered saline (PBS) control (Fig. 1B) (P value of 0.0014 by analysis of variance [ANOVA], P value of <0.05 for PBS versus 4C5, nonsignificant P value for 19A10, P value of <0.001 for IgG control versus 4C5, and P value of <0.05 for 19A10), we observed deposition of the membrane attack complex (MAC) in K1 K. pneumoniae bacteria only after binding of 19A10 (Fig. 1C). Neutrophils can release web-like structures, called neutrophil extracellular traps (NETs), that trap and kill microbes (19). This process is called NETosis and can enhance the inflammatory response and clearance of microbes. We found K1 K. pneumoniae coincubated with either MAb 4C5 or 19A10 led to a significant release of NETs similar to NETs induced by hyphae of Candida albicans (20) (see Fig. S1 in the supplemental material). NETs were not induced by IgG control Ab or PBS (19).

**4C5 and 19A10 are opsonophagocytic Abs.** Promoting opsonophagocytic efficacy is considered a major predictor of antibody protective efficacy. Accordingly, MAb coincubation decreased K1 K. pneumoniae virulence in the Galleria mellonella model, which relies on phagocytic cells as its major host defense (P value by log-rank test of...
0.0172 and 0.032 for 4C5 and 19A10, respectively) (Fig. S2). Accordingly, both 4C5 and 19A10 also promote uptake of K1 \textit{K. pneumoniae} cells by primary murine macrophages. Specifically, opsonization with 4C5 and 19A10 significantly enhanced phagocytosis (22.5% and 42.4% for 4C5 and 14.9% and 25.5% for 19A10) (\(P\) value of \(0.0022\) by \(2\)-way ANOVA and \(P\) values of \(0.0022\) and \(0.0001\) for PBS versus 4C5) (Fig. 2A and Movie S1b and c) at 67 and 105 min after infection. We evaluated if Fc-receptor engagement was required for phagocytosis. We found that phagocytosis was diminished in macrophages from Fc \(R\)-deficient mice (Fig. 2A) (\(P\) value of \(0.0001\) by \(2\)-way ANOVA, \(0.0001\) for both time points for 4C5, and \(0.0022\) and \(0.0001\) for 19A10 for each time point). Similar opsonophagocytic efficacy of the MAbs was confirmed with THP-1, a human monocyte cell line (Fig. 2B). Killing experiments performed in J744.16 cells demonstrated that killing occurred only in MAb-treated macrophages (Fig. S2B and C and Movie S1a) and not in control experiments.

**FIG 1** (A) Agglutination of \textit{K. pneumoniae} serotype K1 (K1-Kp) in the presence of 10 \(\mu\)g of MAb. (Lower) Agglutination of the GFP-labeled \textit{K. pneumoniae} strain. (B) Fluorescence signal of anti-human C3c fluorescein isothiocyanate (FITC) was measured in K1 \textit{K. pneumoniae} bacteria incubated with MAbs and PBS. The mean percent increase of FITC signal relative to each PBS control is shown with standard deviations. (C) Binding of anti-human C5b-9 was measured by ELISA in K1-K. pneumoniae bacteria incubated with NHS or Hi-NHS and MAbs, aggregated human IgG, or PBS. Mean percent increase of AP signal relative to each PBS control is shown with standard deviations from 3 replicates. \(P\) values were calculated by ANOVA with post hoc Tukey’s multiple-comparison test. (D) Quantitation of NET release by human neutrophils after stimulation with \textit{C. albicans}, \textit{K. pneumoniae}, or \textit{K. pneumoniae} incubated with MAbs 4C5 and 19A10 and an IgG control or unstimulated neutrophils (NE). Bars reflect mean percentages (with standard deviations) of neutrophil nuclei with an area exceeding 1,000 \(\mu\)m\(^2\) over the total neutrophils in the field (3 independent replicates). \(P\) values were determined by one-way ANOVA with Sidak’s multiple-comparison test. *, \(P < 0.05\); ***, \(P < 0.001\); ****, \(P < 0.0001\).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Affinity and germ line VR gene usage of MAbs 4C5 and 19A10</th>
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<tr>
<td>MAb</td>
<td>(K_d) (nM)</td>
</tr>
<tr>
<td>4C5</td>
<td>0.36 ± 0.072</td>
</tr>
<tr>
<td>19A10</td>
<td>0.24 ± 0.032</td>
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\(K_d\), dissociation constant.  
\(\mu\)GT/junction analysis gives no results for this junction.
Treatment with 4C5 and 19A10 separately and in combination enhances survival of K1 K. pneumoniae-infected mice. Protective efficacy next was investigated in vivo in two different murine infection models using K1 K. pneumoniae strain 20. For these experiments, Swiss-Webster mice were pretreated with PBS, 500 μg of an IgG isotype control, 4C5, 19A10, or a combination of both MAbs (each at 250 μg). Treatment with 4C5 significantly enhanced survival compared to that for PBS- or sham-treated mice (P value of 0.0108) (Fig. 3A). MAb-treated mice also exhibited reduced bacterial burden (CFU) in liver, lung, and spleen compared to control-treated mice (P value of <0.0001 by ANOVA; P value for 4C5 of <0.0001) (Fig. 3B). For mice treated with 19A10, a similar protective trend (P = 0.068) and a significantly reduced bacterial organ burden (P value of <0.0001) were observed. Importantly, treatment with combinations of MAbs (250 μg each) enhanced protective efficacy even further (P value of 0.0024).
and also reduced bacterial burden significantly (P value of <0.0001). When MAb doses were lowered to 50% (250 μg, 12.5 mg/kg of body weight), similar protection was seen (Fig. S3A and B).

Similar trends were observed in an intratracheal infection (i.t.) model where mice were treated intravenously (i.v.) with PBS, isotype control, or MAbs alone or in combination 24 h before intratracheal inoculation of 10⁴ CFU of K1 K. pneumoniae. Treatment with 4C5 exhibited superior protective efficacy in survival compared to 19A10 treatment (Fig. 3C) (P value for 4C5 treatment, 0.0048; P value for 19A10 treatment, 0.0244) and decreasing bacterial burden in organs (Fig. 3D) except in lungs. Survival (combination P value of 0.0116) as well as bacterial clearance were further enhanced in this model by treatment with MAb cocktail.

Cytokine levels of gamma interferon (IFN-γ), interleukin-1β (IL-1β), IL-2, IL-6, and tumor necrosis factor alpha (TNF-α) in liver and spleen homogenates of MAb-treated mice were significantly lower than those of sham-treated mice (P value of <0.0001 by 2-way ANOVA for both liver and spleen) (Fig. S3C and D). Similar trends were noted in lungs (Fig. S3E).

Treatment with MAbs reduces ampicillin-induced dissemination from gut in K1 K. pneumoniae-colonized mice. Protective efficacy of MAbs was examined in a murine model of gastrointestinal colonization, where bacterial dissemination to liver, spleen, and mesenteric lymph nodes (MLN) is promoted by treatment with antibiotics. Mice colonized with 10⁸ CFU of green fluorescent protein (GFP)-labeled K1 K. pneumoniae via oral ingestion were given ampicillin, leading to a significant increase in bacterial burden in feces and subsequent translocation and dissemination to MLN, spleen, and liver (Fig. 4A to C). We investigated if MAb treatment protected against dissemination and decreased bacterial burden in MLN, spleen, and liver (Fig. 4B). Our data indicate that bacterial dissemination to the MLN, liver, and spleen was significantly decreased and bacterial burden was up to 6 logs lower in those organs compared to the PBS-treated mice (Fig. 4C) (P value of <0.0001 by 2-way ANOVA). In contrast, bacterial colonization seen in feces showed no significant differences with MAb treatment, indicating that colonization per se is not affected by systemic MAb treatment.

Antibodies promote capture of K1 K. pneumoniae in the liver. Kupffer cells in the liver are resident professional phagocytes and constitute a key component of the unique hepatic innate immune system, which is designed as a barrier between the digestive system and the rest of the body. Intravital microscopy (IVM) allows visualization of dynamic events in the hepatic microvasculature when mice are i.v. injected with 10⁸ CFU of GFP-labeled kKp. Time-lapse video demonstrated significantly higher numbers of captured bacteria and decreased presence of bacteria flowing through blood vessels when mice were treated with K1-specific MAbs (P value of <0.0001 by ANOVA; MAb treatment had different P values of <0.05 at different time points) (Fig. 5A and B; Video 5).
S2a and b) compared to control-treated mice (Video S2c). When lower numbers of bacteria (5 × 10^4 CFU) were injected intraperitoneally (i.p.) with K1-specific MAbs, K1 K. pneumoniae bacteria were completely cleared. No bacteria was detected in the blood vessels or captured in the tissue (P < 0.001 and P = 0.0014, respectively, by ANOVA) (Fig. S4A and B, Video S3a and b). Without concomitant MAb treatment, K1 K. pneumoniae bacteria were detected abundantly in the abdominal cavity (Fig. 5C; Fig. S4C and Video S3c). In summary, these findings demonstrate that MAb treatment enables the liver to be an effective barrier because of efficient opsonophagocytic action promoted by K1-specific MAb treatment.

DISCUSSION

Polysaccharide capsules of diverse microbes elicit protective immune responses in the host (21, 22). Clinical data from patients with recurrent hvKp-mediated liver abscesses indicate that opsonizing and killing efficacy of serum increases over time, consistent with an evolving protective antibody response (23). We now present two lead MAbs that control invasive infections caused by an hvKp strain.

Common protective characteristics were observed with both MAbs, although they differ in isotype and bind distinct nonoverlapping epitopes. The capsule of hvKp strains evades the human immune response by inhibiting binding of complement, which can kill on its own or promote phagocytosis (24). Only IgG3 MAb 19A10 elicited enhanced terminal complex (MAC) formation, although both MAbs promoted C3c deposition. Isotype-specific differences in complement activation and protective efficacy have been described (25, 26). Also, others have shown that IgG1 MAbs can be highly protective anti-CPS MAbs and exhibit superior FcγR activation signaling compared to IgG3 Abs (27), which could explain higher efficacy even in the context of comparable binding affinities. Ultimately, experiments with isotype switch variants are warranted to optimize isotype choice prior to MAb humanization.

Ab-opsonized K1 K. pneumoniae is predominantly FcγR mediated. Ab-opsonized Salmonella escapes from the phagolysosome (28); under our experimental conditions we cannot determine if MAbs promote phagolysosomal escape, as they are already killed. Protection was demonstrated in two murine infection models, and consistent with our in vitro data, we found 4C5 was superior to 19A10. Although we found an incremental increase of the bacterial burden with the use of combination therapy compared to the use of each MAb alone, our data also show that combining MAbs, which mimics a protective polyclonal host immune response, can enhance that passive immunotherapy.

Protective efficacy of MAbs in a colonization model supports a novel indication for antimicrobial MAbs. Klebsiella infections in the liver originate from the gut, which is colonized with the bacteria in a significant percentage of Asian patients (29). It has been
shown that in colonized mice ampicillin use increases the risk of hvKp-induced liver abscess (30). Thus, it is reasonable to expect similar risks in humans who are treated with antibiotics that cause major qualitative changes in the gut microbiome. We demonstrate in the event of dissemination from the gut that the number of bacterial CFU in MLN, spleen, and liver was significantly decreased when mice were pretreated with CPS-specific MAbs. This finding also has potential implications for preventing infections in patients colonized with carbapenem-resistant K. pneumoniae strains (ST258 clone), which result in disease in 50% of cases (31, 32). Based on our data, we propose that these patients could receive prophylactic MAb therapy that is specific for the capsules of those strains prior to receiving broad-spectrum antibiotics to prevent dissemination and disease.

Intravital microscopy investigates in real time the host-pathogen interaction in liver sinusoids (3), which are lined by phagocytic Kupffer cells that create a highly efficient filtering system. Altered cytokine expression in tissue of treated cells may be the result of MAb-modulated phagocyte-K1 K. pneumoniae interaction, which could further alter recruitment of neutrophils as described in other infections (33). During overwhelming infection or in the microenvironment of an abscess, phagocytosis alone may not be sufficient to clear an infection. Murine infection models show that mice with impaired capacity to form NETs are more prone to K. pneumoniae infections, indicating that NETs are relevant for successful clearance (34). Our observation that K1-specific MAbs promote NETosis is therefore consistent with these data and suggests additional mechanisms for MAb-based protection are operative. We propose that specific MAb agglutinates K. pneumoniae, cross-links Fc receptors, and promotes NET release.

Compared to triumphs seen with antitumor and anti-inflammatory MAb treatment, progress with anti-infective MAb development regrettably lags behind. This is surprising given the protective efficacy of hyperimmune-globulin preparations (35) and the overwhelming evidence with anti-infective Abs. They can work (36) as monotherapy, cocktails, or in combination with antimicrobial drugs (37). One problem may be that MAb cocktails commonly are more efficacious than monotherapy, especially if rapidly expanding pathogen populations are to be contained. With revised FDA licensing rules for MAbs, it has become easier to receive FDA approval for cocktails. Accordingly, MAb cocktails for treatment of Clostridium difficile, rabies prophylaxis (38, 39), and Ebola virus (40) are now being tested in advanced clinical trials. Another obstacle is strain variability, which is inherently underestimated by research with laboratory-adapted strains. This is also an especially major challenge for K. pneumoniae strains of the ST258 clonal background, as their CPS is very heterogeneous. Both cross-reactive MAbs (13) as well as MAbs recognizing more universal targets can be developed to overcome this barrier. Recent work with MAbs to the polysaccharide poly-(β-1,6)-N-acetylglucosamine are encouraging (41), and future studies should be done comparing efficacy of MAbs with differences in degree of microbial specificity. The spread of multidrug-resistant (MDR) bacteria in the community as exemplified now by MDR hvKp is a crucial development, associated not only with increased morbidity, mortality, and health care costs but also with inappropriate antibiotic use (42). Our findings may have broader implications, because treatment with anti-infective MAbs against a gut-colonizing bacteria like hvKp is a targeted intervention that should not disturb the microbiome, promote resistance, or lead to emergence of other serotypes. Our data support efforts to develop anti-infective antibodies, which, as evidenced by the efficacy of palivizumab, a MAb-based therapy against respiratory syncytial virus (RSV), in preterm infants, will even be effective in immunocompromised hosts. With continuous fast-moving globalization and unrestrained antibiotic usage, MDR bacteria are likely to become an increasing problem.

**MATERIALS AND METHODS**

K1 K. pneumoniae strains. K1 K. pneumoniae strains (n = 4) were collected from patients at Montefiore Medical Center (MMC) and Stony Brook University (SBU) Hospital. One strain was kindly provided by Thomas Russo at the University at Buffalo, SUNY. K. pneumoniae strains were cultured in Luria-Bertani (LB) broth or agar plates at 37°C. K. pneumoniae strain 20, of clonal sequence type ST23 that
expressed K1-CPS, was used for CPS purification and for in vitro and in vivo experiments (13). The strain was GFP labeled with pPROBEKT plasmid kindly provided by E. W. Triplett (43).

**CPS purification and conjugation.** K1-CPS was isolated for conjugation with protective antigen (PA) from *Bacillus anthracis* (see Methods S1 in the supplemental material). For conjugation, K1-CPS was activated with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as described previously, with modifications (13, 44) (Methods S1).

**MAb generation and characterization.** MAbs to K1-CPS were generated by immunization of BALB/c mice with 100 μg of PA-conjugated K1-CPS in complete Freund’s adjuvant followed by boosters of PA-conjugated K1-CPS in incomplete Freund’s adjuvant. Fusion and cloning were performed as described previously (45). GenScript sequenced the variable region (VR) of 19A10- and 4C5-generated MAbs, which were analyzed using International ImMunoGeneTics (IMGT) Information System software. MAb affinity was determined by enzyme-linked immunosorbent assay (ELISA); briefly, decreasing concentrations of the MAbs were incubated for 1 h at 37°C on a 0.5 μg/ml K1-CPS-coated plate, and the binding was detected with the appropriate alkaline phosphatase (AP)-labeled secondary MAb.

**Agglutination assays.** Agglutination was carried out on glass slides as described previously (21). Briefly, 100 μl of PBS with 3 × 10⁵ CFU of *K. pneumoniae* was incubated with or without the MAbs (20 μg) for 1 h at room temperature. Agglutination was observed under the microscope at ×200.

**C3 and MAC complement deposition assays.** Deposition of C3c was measured by flow cytometry as described previously (46) (Methods S1). For MAC deposition assays, an overnight culture of serotype K1 was resuspended (2 × 10⁵ CFU of K1) in 100 μl of normal human serum (NH5) or heat-inactivated NHS (HI-NHS) (56°C for 1 h). Ten micrograms of either aggregated human IgG (classical positive control for classical pathway), IgG control, 4C5, or 19A10 then was added, incubated for 1 h at 37°C, and washed 2× with buffer. Pellets were resuspended with biotin-labeled anti-human C5b-9 (Quidel) (1:500 dilution) and incubated for 1 h at 37°C. After resuspended washing, streptavidin-AP (Vectastain ABC-AP) was added and incubated for 30 min at room temperature. After washing, pellets were resuspended in AP-substrate buffer and the A₆₅₀ was measured. Results are expressed by the increment of the AP signal of MAb treatment with respect to PBS incubation.

**Galleria mellonella virulence assays.** Virulence of K1 *K. pneumoniae* was assessed in *G. mellonella* as previously reported (13) (Methods S1).

**Phagocytosis experiments.** The J744.16 murine macrophage cell line, THP-1 human monocyte cell line, and murine primary bone marrow macrophages were used for phagocytosis assays with bacteria labeled by pHrodo (Life Technologies). THP-1 cells (and J744.16 cells), differentiated with phorbol 12-myristate 12-acetate (PMA), were seeded at a concentration of 5 × 10⁴ in 35-mm glass-bottom dishes (MatTek Corporation) and incubated with pHrodo-labeled K1 *K. pneumoniae* cells for 1 h with PBS and with 20 μg of isotype control, 4C5, or 19A10 MAb at a multiplicity of infection of 10:1. Phagocytosis was immediately assessed using a Zeiss Axiosvert 200M inverted microscope with a 10× objective with the dish housed in an enclosed chamber under conditions of 5% CO₂ and 37°C. Images were taken every 3 min for the 2-h duration of the experiment. Phagocytosis was measured by counting the percentage of macrophages that display labeled bacteria at 67 and 105 min. Primary bone marrow macrophages were isolated as described previously (47) from wild-type or FcR knockout (KO) mice (FcεRI−/−; The Jackson Laboratory). After 7 days of macrophage differentiation, phagocytosis experiments were carried out as outlined above. Three independent experiments were performed. *In vitro* killing of K1 *K. pneumoniae* was investigated in the J744.16 macrophage cell line as previously reported (48) (Methods S1).

**NETosis experiments.** NETosis assays were carried out with human neutrophils as described previously (20), with modifications (Methods S1).

**In vivo mouse experiments.** Six- to 8-week-old female Swiss Webster mice (Taconic Biosciences; n = 10) were injected intraperitoneally (i.p.) with PBS, with 500 μg or 250 μg of control isotype, 4C5, and 19A10, or with 250 μg or 125 μg of 4C5 and 19A10 MAbs 120 min prior to challenge with 5 × 10⁸ CFU of K1 *K. pneumoniae* i.p. (sepsis model) or were treated i.p. 24 h prior to intratracheal (i.t.) challenge with 10⁵ CFU of K1 *K. pneumoniae* (pulmonary infection model). Survival of 6 mice was monitored for 15 days. Liver, spleen, and lungs of 4 mice were processed to enumerate bacteria in homogenized tissue. Cytokine levels in homogenized tissue were measured using commercial ELISA kits. Gastrointestinal colonization was performed as described previously, with modifications for *K. pneumoniae* (49). Briefly, mice were deprived of food and water for 4 h prior to infection. Individually housed mice then were given a 0.5-cm² piece of bread inoculated with 1 × 10⁸ CFU of GFP-labeled K1 *K. pneumoniae* in PBS. On day 3 postinfection, mice were provided with water supplemented with 0.5 g/liter of ampicillin for the duration of the experiment. In addition, on days 3 and 4 mice received a dose of PBS, 500 μg of 4C5 and 19A10, or 250 μg of 4C5 and 19A10 i.v. On day 8, mice were euthanized and organs and tissues were prepared as previously described (49).

**IVM.** A liver intravalvular protocol was carried out as previously described (50) (Methods S1).

**Statistical analysis.** Statistical tests were performed with GraphPad Prism 6 for Mac. For multigroup comparisons of parametric data (e.g., phagocytosis, log-transformed CFU, and complement deposition), ANOVA with post hoc analysis using Tukey’s, Sidak’s, or Dunnett’s comparison test was used. For two-group comparisons of parametric data (log-transformed CFU), paired t tests corrected for multiple comparisons using the Holm-Sidak method were performed. Survival analysis was performed using a log-rank (Mantel-Cox) test corrected with Bonferroni’s multiple-comparison test.

**Ethics statement.** Animal study protocols were approved by the Animal Committee (IACUC) at Stony Brook University, approval number 628253, and by the Animal Committee (IACUC) at the University of...
Maryland, approval number 340281. The study was performed in strict accordance with federal, state, local, and institutional guidelines that include the Guide for the Care and Use of Laboratory Animals (51), the Animal Welfare Act, and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (52). All surgery was performed under ketamine and xylazine anesthesia, and every effort was made to minimize suffering. Human studies were approved by the Stony Brook University Human Subjects Committee (IRB), approval number 718744. Healthy donors gave written informed consent for blood extraction.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00456-16.

REFERENCES


