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RESEARCH ARTICLE

Serum Antibody Responses against Carbapenem-Resistant *Klebsiella pneumoniae* in Infected Patients

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ABSTRACT Capsular polysaccharide (CPS) heterogeneity within carbapenem-resistant *Klebsiella pneumoniae* (CR-*Kp*) strain sequence type 258 (ST258) must be considered when developing CPS-based vaccines. Here, we sought to characterize CPS-specific antibody responses elicited by CR-*Kp*-infected patients. Plasma and bacterial isolates were collected from 33 hospital patients with positive CR-*Kp* cultures. Isolate capsules were typed by *wzi* sequencing. Reactivity and measures of efficacy of patient antibodies were studied against 3 prevalent CR-*Kp* CPS types (*wzi29*, *wzi154*, and *wzi50*). High IgG titers against *wzi154* and *wzi50* CPS were documented in 79% of infected patients. Patient-derived (PD) IgGs agglutinated CR-*Kp* and limited growth better than naive IgG and promoted phagocytosis of strains across the serotype isolated from their donors. Additionally, poly-IgG from *wzi50* and *wzi154* patients promoted phagocytosis of nonconcordant CR-*Kp* serotypes. Such effects were lost when poly-IgG was depleted of CPS-specific IgG. Additionally, mice infected with *wzi50*, *wzi154*, and *wzi29* CR-*Kp* strains preopsonized with *wzi50* patient-derived IgG exhibited lower lung CFU than controls. Depletion of *wzi50* antibodies (Abs) reversed this effect in *wzi50* and *wzi154* infections, whereas *wzi154* Ab depletion reduced poly-IgG efficacy against *wzi29* CR-*Kp*. We are the first to report cross-reactive properties of CPS-specific Abs from CR-*Kp* patients through both *in vitro* and *in vivo* models.

IMPORTANCE Carbapenem-resistant *Klebsiella pneumoniae* is a rapidly emerging public health threat that can cause fatal infections in up to 50% of affected patients. Due to its resistance to nearly all antimicrobials, development of alternate therapies like antibodies and vaccines is urgently needed. Capsular polysaccharides constitute important targets, as they are crucial for *Klebsiella pneumoniae* pathogenesis. Capsular polysaccharides are very diverse and, therefore, studying the host's capsule-type specific antibodies is crucial to develop effective anti-CPS immunotherapies. In this study, we are the first to characterize humoral responses in infected patients against carbapenem-resistant *Klebsiella pneumoniae* expressing different *wzi* capsule types. This study is the first to report the efficacy of cross-reactive properties of CPS-specific Abs in both *in vitro* and *in vivo* models.

KEYWORDS carbapenem-resistant *Klebsiella pneumoniae*, capsular polysaccharide, *wzi* capsule, patient-derived antibodies, CPS-specific antibodies, CPS-specific antibody, *Klebsiella pneumoniae*, carbapenem resistant, host-pathogen interactions, humoral immunity, patient-derived antibody, *wzi* typing

Infections with carbapenem-resistant *Klebsiella pneumoniae* (CR-*Kp*) are associated with high mortality rates (1). Thus, the World Health Organization (WHO) considers the development of novel therapeutics for CR-*Kp* a critical priority (2). A large prospective observational multicenter study demonstrated that the spread of CR-*Kp* in the

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Interesting study by @kasturi1990 et al. in @BettinaFries lab at @stonybrookmedicine shows cross-reactive properties of Klebsiella pneumoniae's CPS-specific antibodies in both in vitro and in vivo models.

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United States is largely driven by the expansion of sequence type 258 (ST258) or related clonal lineages (clonal group 258 [CG258]) of CR-*Kp*. In that study, CG258 represented 74% of carbapenemase-producing *K. pneumoniae* isolates (3). The genome of ST258 strains is highly conserved, with two dominant subclades (clade 1 and clade 2) differing only by several hundred kilobases, and *wzi* gene-based capsular typing has shown the predominance of only a few capsular polysaccharide (CPS) subtypes (4–6). Specifically, clade 2 strains almost exclusively produce *wzi154* CPS, whereas clade 1 strains chiefly produce *wzi29* and *wzi50* capsules, but can produce other capsular types as well (7, 8). Importantly, infection with *wzi154*/clade 2 strains has been associated with worse prognoses than infections by other strains (8–11).

The polysaccharide capsule of *K. pneumoniae* protects the pathogen from host cell-mediated killing and the bactericidal effect of serum, thus contributing to *K. pneumoniae* pathogenicity (12). Studies examining the efficacy of antibodies (Abs) directed against CR-*Kp* CPS have demonstrated their potency in human cells and animal models (13–17), and CPS vaccines have the potential to prevent or moderate the severity of CR-*Kp* infection in monkeys and decrease the prevalence and emergence of resistant strains (14, 18–20). However, it is unknown if natural antibody responses to *K. pneumoniae* rely on anticapsular responses. Given the heterogeneity of the CPS, it is important to understand whether diverse *wzi* CPS types are recognized differentially by the immune system during an infection. Furthermore, it is still unknown whether anti-CPS antibodies cross-react and cross-protect across clades that express CPS with different *wzi* types.

To understand whether vaccination with CPS constitutes a feasible strategy to elicit a protective immune response and mitigate CR-*Kp* emergence, we characterized the humoral response in a cohort of hospitalized patients infected or colonized with CR-*Kp*. We compared the Ab response elicited by different *wzi* capsule types (*wzi29*, *wzi154*, and *wzi50*) and assessed the protective opsonophagocytic efficacy of human anti-CPS polyclonal IgG against CR-*Kp in vitro* and *in vivo*. This study is the first to our knowledge to document anti-CPS humoral responses in patients infected with different CR-*Kp* strains that produce specific *wzi*-type CPS. Our data indicate for the first time a cross-reactive therapeutic potential of the *wzi50* anti-CPS Abs. The implications of these findings for efforts to developing anticapsular therapeutics are discussed in this study.

(These data were presented in part at IDWeek 2019–IDSA, 2 to 6 October 2019, Washington, DC [21].)

RESULTS

Demographics and clinical variables. We identified 36 patients who were hospitalized at Stony Brook University Hospital (SBUH) between 2017 and 2019 with bacterial cultures that grew CR-*Kp*. Plasma samples were obtained from 33, while 3 did not give consent. Of the 33 who consented, 23 met published criteria for "symptomatic infection with CR-*Kp*," while 10 were classified as "asymptomatic infection or colonized with CR-*Kp*" (11). Both cohorts (infected and colonized with CR-*Kp*) were similar in age and gender distribution, but infected patients had a median length of stay (LOS) of 20.5 days (interquartile range [IQR] = 14 to 52.5 days; $P < 0.0001$), whereas colonized patients had a median LOS of 17 days (IQR = 8 to 33 days; $P = 0.0003$). Subsequently, the median time to first positive culture from the day of admission was longer for infected patients compared to colonized patients (7.5 versus 3 days, respectively). Capsular serotyping by *wzi* sequencing showed 12 isolates to be *wzi154*, 10 to be *wzi29*, 2 to be *wzi50*, and 9 to be of other *wzi* types (Table 1). *wzi154*, *wzi29*, and *wzi50* were previously identified as the most common *wzi* types among ST258 isolates in the New York City area (7).

Anti-CPS antibody responses in clinical patient samples. Patient Ab responses to *wzi29*, *wzi54*, and *wzi50* CPS were measured by enzyme-limited immunosorbent assay (ELISA) and indicated the highest overall IgG responses to *wzi50* CPS, followed by those to *wzi154* CPS. We also observed minimal IgG response to *wzi29* CPS and that 7 plasma

TABLE 1 Patient characteristics

Characteristic	Infected	Colonized
No. (%) of consenting patients	23 (69.7)	10 (30.3)
Median age (yrs)	70 (37 to 86 yrs)	66.5 (23 to 88 yrs)
Gender (*n*)		
Male	13	7
Female	10	3
Median (range) length of hospital stay (days)	20.5 (5 to 262)	17 (4 to 99)
Median no. (range) of hospital days blood culture was positive for CR-*Kp*	7.5 (0 to 145)	3 (0 to 80)
Source (*n*) of isolates	Urine (9), blood (4), respiratory (5), other (abdominal fluid [4], penial wound [1])	Urine (7), blood (0), respiratory (2), other (rectal swab [1])
wzi types (*n*)		
wzi29	7	3
wzi154	7	5
Other	*wzi26* (1), *wzi101* (1), *wzi50* (1), *wzi60* (1), *wzi96* (1), *wzi173* (3), *wzi355* (1)	*wzi7* (1), *wzi50* (1)

samples lacked any CPS reactivity (Fig. 1A). IgM and IgA titers showed similar trends (see Fig. S2 in the supplemental material). The magnitudes of IgG, IgM, and IgA responses against *wzi*29 and *wzi154* were comparable regardless of the state of CR-*Kp* infection ("infected" versus "colonized"). In contrast, higher IgA titers against *wzi50* CPS were only observed in symptomatic patients (Fig. S1). Plasma ($n = 33$) was also tested for anti-lipopolysaccharide (anti-LPS) Abs, which have also been observed to be protective (22). Both cohorts exhibited high IgG and low IgA and IgM reactivity against LPS in plasma (Fig. 1B).

Importantly, no Ab reactivity with CPS or LPS was detected in the plasma of healthy individuals, thus suggesting Ab responses were elicited during the recent CR-*Kp* infection (Fig. 1A and B).

Knowing the *wzi* type of the CR-*Kp* strains infecting the patients permitted further subanalysis of Ab reactivity. Notably, no *wzi29*-specific IgG was detected in the plasma of patients infected with *wzi29* CR-*Kp* (Fig. 1C), although those patients produced cross-reactive Abs that bound to *wzi154* (50%) and *wzi50* (88%) CPS (Fig. 1C). While 50% of patients infected with *wzi154* CR-*Kp* mounted an Ab response to *wzi154* CPS, 67% of those patients also exhibited cross-reactive IgG Abs to *wzi50* CPS (Fig. 1C). Similarly, while 81% of patients infected with CR-*Kp* strains with other *wzi* CPS (including both *wzi50*-infected patients) produced IgGs that recognized *wzi50* CPS, only low Ab titers binding *wzi29* and *wzi154* CPS were observed (Fig. 1C). No IgM reactivity to any CPS type were observed in patients infected with *wzi29* CR-*Kp* (Fig. S2A), whereas few patients infected with CR-*Kp* strains with *wzi29*- and *wzi154*-type CPS had low IgA titers to *wzi29*-type CPS (Fig. S2B). Lack of any IgG response to *wzi29*-type CPS raised the concern that the immunogenic epitopes were destroyed during purification, and therefore ELISA was done with whole bacteria, which confirmed that most patients' plasma bound to whole *wzi29* MMC36 bacteria (Fig. 1D). Isotype subclass analysis showed that anti-CPS Abs included IgG2, IgG3, and IgG4 isotypes, whereas anti-LPS Abs were all IgG2 (Fig. S3).

Cross-agglutination and serum killing of CR-*Kp* by patient-derived IgGs. To further explore anticapsular antibody immunity against CR-*Kp*, we examined agglutination and serum resistance of bulk IgG purified from the plasma of a patient who possessed high anti-CPS titers (optical density [OD] > 0.6). First, we tested the ability of poly-IgG$^{\#168}$ (purified from a patient infected with *wzi50*-producing SBU168) to agglutinate CR-*Kp* strains with different CPS types (Fig. 2A and B). Our data showed that poly-IgG$^{\#168}$ agglutinated CR-*Kp* strains MMC34 (*wzi154*), MMC36 (*wzi29*), and MMC38 (*wzi50*) (Fig. 2A). The extent of agglutination (average area of

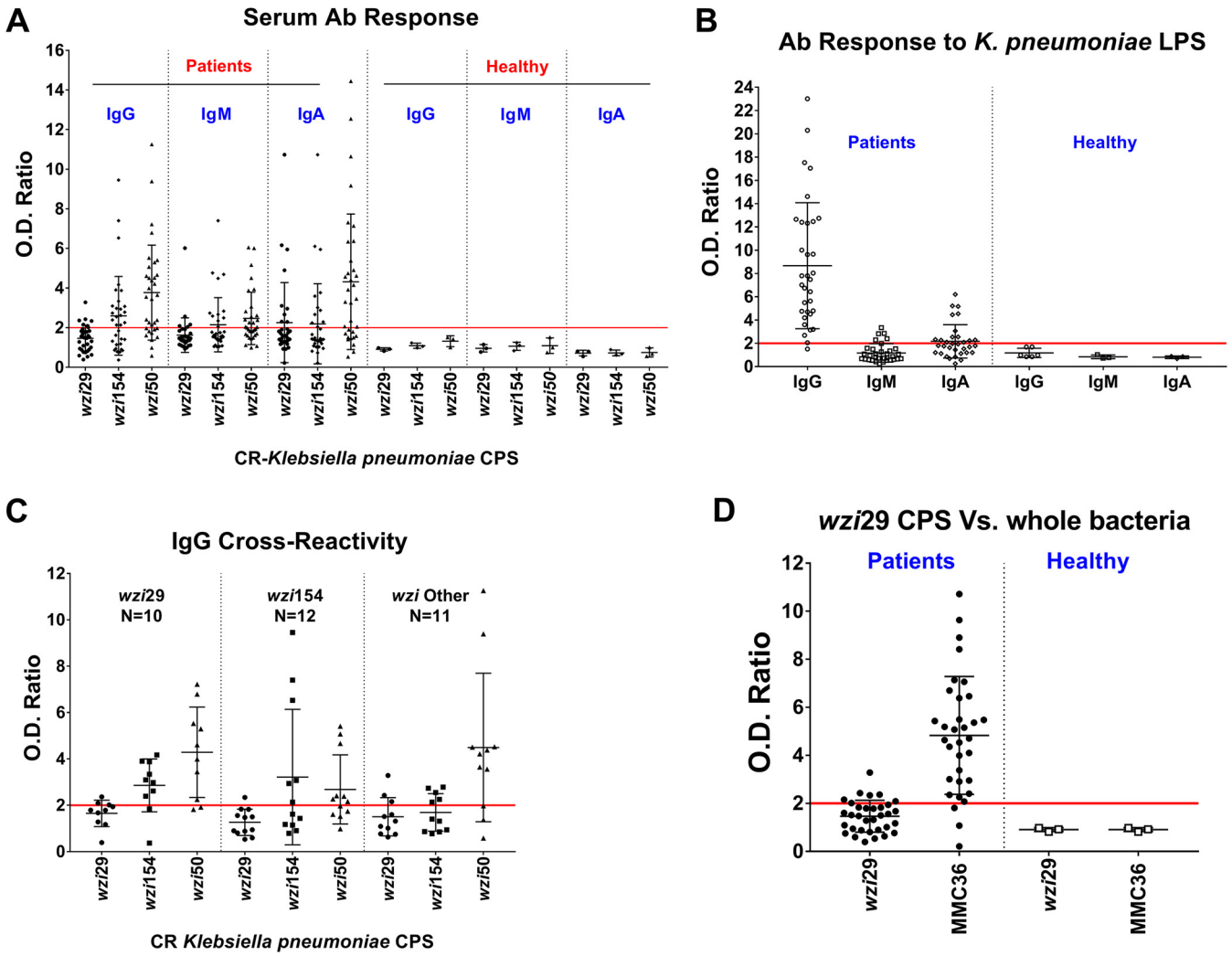

FIG 1 Humoral responses to capsular polysaccharides (CPS) and lipopolysaccharides (LPS) isolated from carbapenem-resistant *K. pneumoniae*. (A) Humoral antibody (Ab) (IgG, IgM, and IgA) responses detected against *wzi29*, *wzi154*, and *wzi50* CPS in carbapenem-resistant *K. pneumoniae* (CR-*Kp*)-infected patients versus healthy controls. (B) Presence of antibodies against *K. pneumoniae* LPS in all CR-*Kp* patients and healthy donors. (C) Antibodies of patients infected with *wzi29 K. pneumoniae* tested against all three CPS types. *wzi154*-infected patients' antibodies tested against all three CPS. Antibodies from patients infected with other *wzi*-type CR-*K. pneumoniae* strains tested against all three CPSs. (D) Presence of antibodies against *wzi29* whole bacteria in CR-*Kp*-infected patient plasma Abs versus healthy donors compared to purified *wzi29* CPS. Each symbol represents one patient. Optical density (OD) ratio = OD CPS/OD bovine serum albumin (BSA). The fold change cutoff is set at $y = 2$ (red line); patients with the presence of anti-CPS Abs in the plasma had values of ≥ 2, whereas patients with undetectable level of anti-CPS Abs in plasma had values of <2. $N = 33$; each dot on the scatterplot represents an individual CR-*Kp* patient's OD ratio, and the deviations in OD ratios are shown as standard deviations (SD).

agglutinated clumps) was quantified by ImageJ software and was found to be significant for strains MMC34 and MMC38 compared to bacteria treated with IgG derived from normal human serum (poly-IgG^{NHS}) (unpaired *t* test, $P = 0.0040$) (Fig. 2B). Next, serum resistance or killing was evaluated over a 3-h incubation time. CR-*Kp* strains differ in their resistance to complement-mediated killing, whereby some strains can be killed, whereas for other strains only growth is inhibited (7). For the complement-sensitive strain MMC34, at 3 h poly-IgG$^{\#168}$ killed 75% of the bacteria ($P < 0.0001$) in 75% normal human serum compared to normal human serum (NHS) and poly-IgG^{NHS} controls (Fig. 2C). Our data indicate that coincubation of poly-IgG$^{\#168}$ significantly impaired the growth of MMC36 in 75% NHS (Fig. 2D) compared to serum with nonspecific poly-IgG^{NHS} ($P < 0.0001$). MMC38 treated with poly-IgG^{NHS} had an overall 145% growth increase, whereas negligible growth was observed in MMC38 treated with poly-IgG$^{\#168}$ during the time interval of 2 and 3 h ($P < 0.0001$) (Fig. 2E).

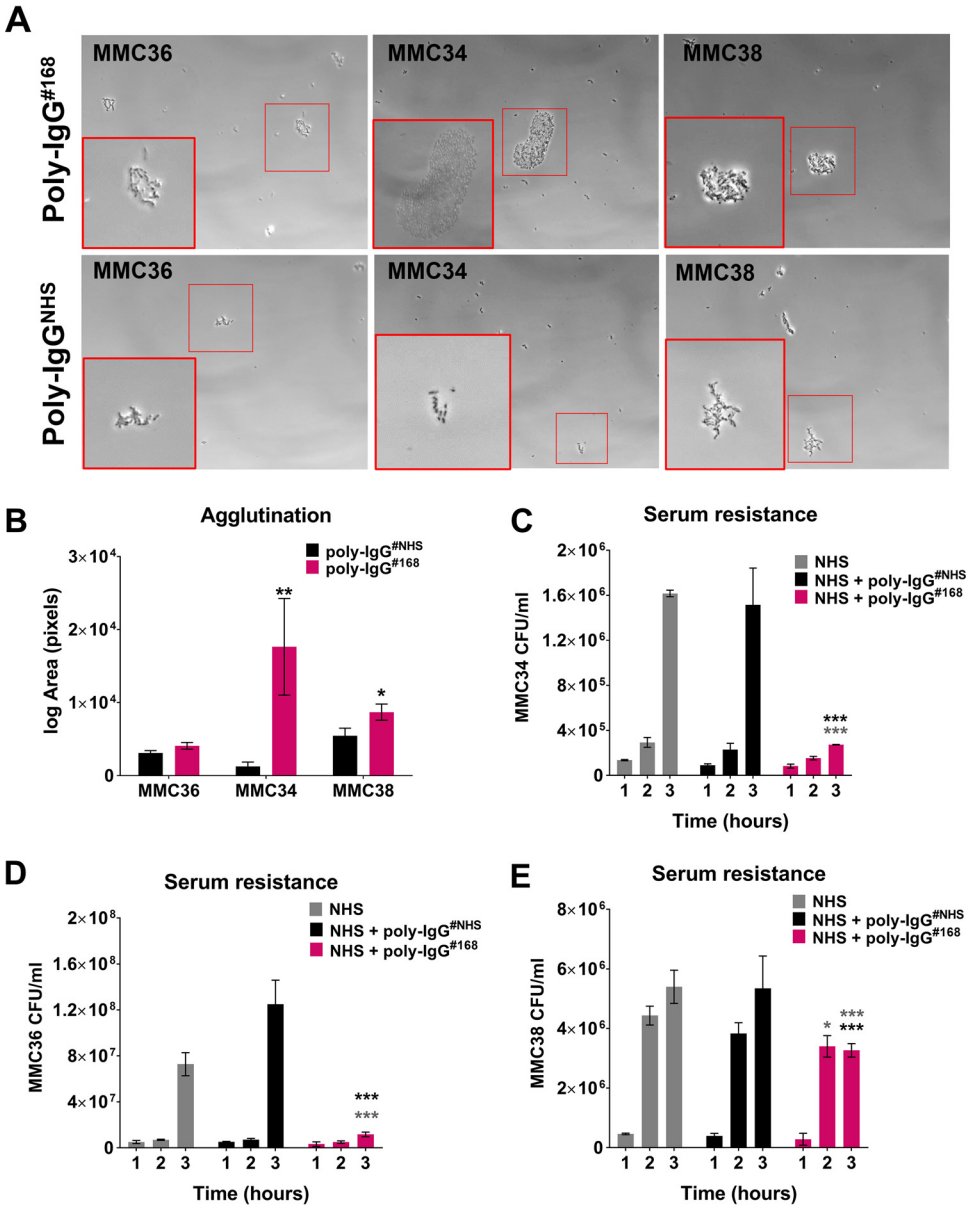

FIG 2 Patient antibodies promote agglutination and decrease serum resistance. (A) *wzi50* patient-derived (PD) IgGs promoted agglutination of *wzi29* MMC36, *wzi154* MMC34, and *wzi50* MMC38 strains, which was visualized with phase-contrast microscopy at ×200 magnification. (B) Quantification of the area of agglutinated bacteria. *wzi50* PD IgGs (poly-IgG$^{\#168}$) (C) mediated serum killing of *wzi154* MMC34, (D) inhibited the growth of *wzi29* MMC36, and (E) inhibited the growth of *wzi50* MMC38 strains, with respect to CR-*Kp* strains treated with poly-IgG$^{\#NHS}$ in 75% natural human serum (NHS). Bars depict means and SD from three independent experiments. Overall differences between treatment groups were determined to be significant by repeated-measures two-way analysis of variance (ANOVA) using Tukey's *post hoc* test, displayed in-graph. For all in-graph statistics, *P* values displayed in gray are comparisons to the NHS-only control group, whereas *P* values in black are comparisons to the poly-IgG$^{\#NHS}$ treated group. *P* values are indicated as "ns" if >0.1, * if < 0.05, ** if <0.01, and *** if <0.001.

Opsonophagocytosis of clinical CR-*Kp* isolates by patient-derived poly-IgG. A key attribute commonly associated with the efficacy of CPS-specific Ab-mediated immunity is opsonophagocytosis (23, 24). To assess the efficacy of different patient-derived (PD) poly-IgGs, we performed opsonophagocytosis experiments in J774 macrophages with Poly-IgG$^{\#207}$, poly-IgG$^{\#219}$, and poly-IgG$^{\#116}$ purified from patients infected with SBU219 (*wzi154*), SBU116 (*wzi50*), and SBU207 (*wzi29*). Opsonophagocytosis of respective CR-*Kp* clinical isolates was promoted in a dose-dependent matter by all 4

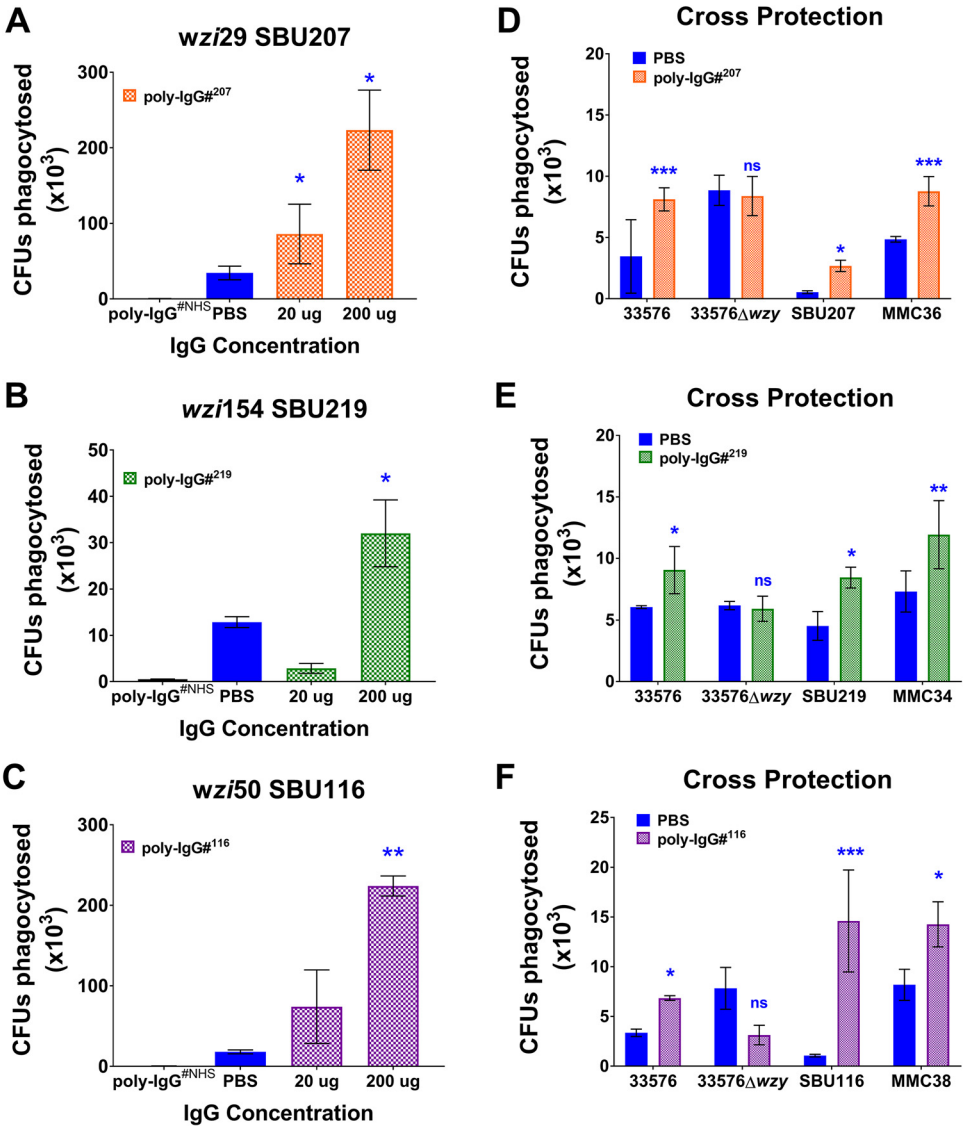

FIG 3 Coincubation with patient-derived IgGs induces opsonophagocytosis of clinical *K. pneumoniae* strains. PD IgGs isolated from patients 207 (*wzi29*), 219 (*wzi154*), and 116 (*wzi50*) induced opsonophagocytosis of patient-matched *K. pneumoniae* (A) SBU207 (*wzi29*), (B) SBU219 (*wzi154*), and (C) SBU116 (*wzi50*) strains, respectively. PD IgGs (D) poly-IgG$^{\#207}$, (E) poly-IgG$^{\#219}$, and (F) poly-IgG$^{\#116}$ broadly recognized and induced opsonophagocytosis of strains with similar *wzi* type and also cross-reacted with 33576 clade 2 *K. pneumoniae* capsular strain but did not mediate phagocytosis of the acapsular 33576 Δ*wzy* mutant strain. Bars depict means and SD from three independent experiments, with wells performed in triplicate. poly-IgG$^{\#NHS}$ and phosphate-buffered saline (PBS) serve as the negative controls for the assay. Phagocytosed bacteria within macrophages were counted by plating lysed macrophages (log CFU phagocytosed) on Luria-Bertani (LB) agar plates. Overall differences between treatment groups were determined to be significant by repeated-measures two-way ANOVA using Tukey's *post hoc* test, displayed in-graph. For all in-graph statistics, *P* values displayed in blue are comparisons to the PBS control. *P* values are replaced with ns if >0.1, * if <0.05, ** if <0.01, and *** if <0.001.

poly-IgGs compared to both poly-IgG$^{\#NHS}$ and phosphate-buffered saline (PBS) controls (Fig. 3A to C and Fig. S4 in the supplemental material). Due to the limited quantity of poly-IgG$^{\#168}$, investigations were continued with poly-IgG$^{\#116}$, which was also derived from a patient infected with *wzi50*-producing CR-*Kp*. Poly-IgG$^{\#207}$ promoted opsonophagocytosis of two different *wzi29* strains, SBU207 and MMC36 (Fig. 3D). In a similar fashion, poly-IgG$^{\#219}$ (Fig. 3E) and poly-IgG$^{\#116}$ (Fig. 3F) induced opsonophagocytic uptake of MMC34 and SBU219 (both *wzi154*); and MMC38 and SBU116 (both *wzi*50) strains, respectively. Interestingly, while poly-IgGs promoted phagocytosis of capsular

strains, including that of 33576 (*wzi154*), it did not mediate phagocytosis of its acapsular 33576 Δ*wzy* mutant (Fig. 3D to F). These data suggest that anti-CPS Abs in PD poly-IgGs convey opsonophagocytic efficacy and also indicate the presence of cross-reactive anti-CPS Abs.

Relevance of anticapsular antibodies for opsonophagocytic efficacy. To test if the protective Abs are anti-CPS, we depleted poly-IgG$^{\#116}$ and poly-IgG$^{\#219}$ of *wzi154* and *wzi50* CPS-specific Abs by coincubation with corresponding CPS-coated beads. Ab-mediated opsonophagocytosis of 4 CR-*Kp* strains producing distinct *wzi* types (*wzi29*, *wzi154*, and *wzi50*) was compared to that of nondepleted poly-IgG$^{\#116}$ and poly-IgG$^{\#NHS}$. Data confirmed significant augmented phagocytosis of all 4 CR-*Kp* strains (Fig. 4A to D) compared to poly-IgG$^{\#NHS}$. For MMC34, MMC38, and SBU116 phagocytosis was significantly lower when PD bulk IgGs were depleted with *wzi154*-CPS-coated beads (92%, 58%, and 44% loss in efficacy, respectively). Whereas, when 116 PD bulk IgGs were depleted with *wzi50*-CPS-coated beads, 94%, 59%, and 50% losses in efficacy were observed for MMC34, MMC38, and SBU116, respectively (Fig. 4A to C). Depletion of CPS-specific Abs from poly-IgG$^{\#116}$ did not affect opsonophagocytosis of the *wzi29* MMC36 strain (Fig. 4D). Similar results were observed when *wzi154*-specific Abs were depleted from bulk poly-IgG$^{\#219}$, resulting in 40% and 30% loss in phagocytic efficacy for MMC34 and MMC38, respectively (Fig. 4E and F), whereas depletion of *wzi50*-specific Abs only affected the phagocytosis of MMC34 (36% loss) (Fig. 4E). Depletion of either anti-CPS Ab did not affect the phagocytosis of MMC36 (data not shown).

Reduced protective efficacy of depleted PD poly-IgG in a murine infection model. Last, we explored if cross-reactive Abs could protect mice from CR-*Kp* infection. We compared CFU in a mouse pulmonary model infected with different CR-*Kp* strains (MMC34, SBU116, and MMC36), preopsonized with either undepleted or depleted poly-IgG$^{\#116}$. Mice injected with poly-IgG$^{\#116}$-opsonized MMC34 exhibited a moderate 1-$\log_{10}$ reduction in bacterial lung burden, as well as a 4-$\log_{10}$ reduction in dissemination to the spleen. (Fig. 5). A 2-$\log_{10}$ and a 1-$\log_{10}$ reduction in lung and spleen CFU, respectively, were seen in mice infected with poly-IgG$^{\#116}$-opsonized SBU116. Depletion of *wzi50*-specific Abs resulted in 1-$\log_{10}$ higher CFU in both lung and spleen compared to undepleted treatment, whereas *wzi154*-specific Ab depletion only affected dissemination to the spleen compared to undepleted treatment (Fig. 5). A significant, albeit small, decrease in lung CFU was observed in mice infected with MMC36 preopsonized with poly-IgG$^{\#116}$, but no dissemination to the spleen was observed in either of the experimental groups. Furthermore, the bacterial burden in the lung was identical between the *wzi154*-depleted group and the PBS control (Fig. 5).

DISCUSSION

Prior studies in mice and monkeys provide compelling evidence that CPS-specific Abs are effective against CR-*Kp* (15, 16, 19, 25). Several groups have developed vaccines that target CPS to prevent *K. pneumoniae* infection (16, 26–28). However, concerns regarding feasibility prevail because of the heterogeneity of the polysaccharide capsule (7). These data constitute the first analysis of the anti-CPS Ab response in CR-*Kp*-infected patients. Several important conclusions can be drawn from our data. First, most CR-*Kp*-infected patients mount a humoral response to the polysaccharide capsule, although it is more variable in its magnitude than Ab reactivity to LPS. Second, CPS-specific Abs cross-react with other capsule types. Third, protective Abs were specific to CPS. Last, poly-IgG from CR-*Kp* infected patients can protect CR-*Kp*-infected mice, and loss of efficacy is observed after depletion of CPS-specific Abs.

Our data represent an unbiased analysis of the Ab response in CR-*Kp*-infected patients, as the cohort included the majority of hospitalized patients diagnosed with CR-*Kp* infection during the studied time period. Although colonized patients were diagnosed earlier and spent less time in the hospital, there were no striking differences with respect to their CPS-specific Ab responses. Although the findings could indicate that the naturally evolving humoral response is not sufficient in eradicating these organisms, this observational study did not systematically assess if colonized patients

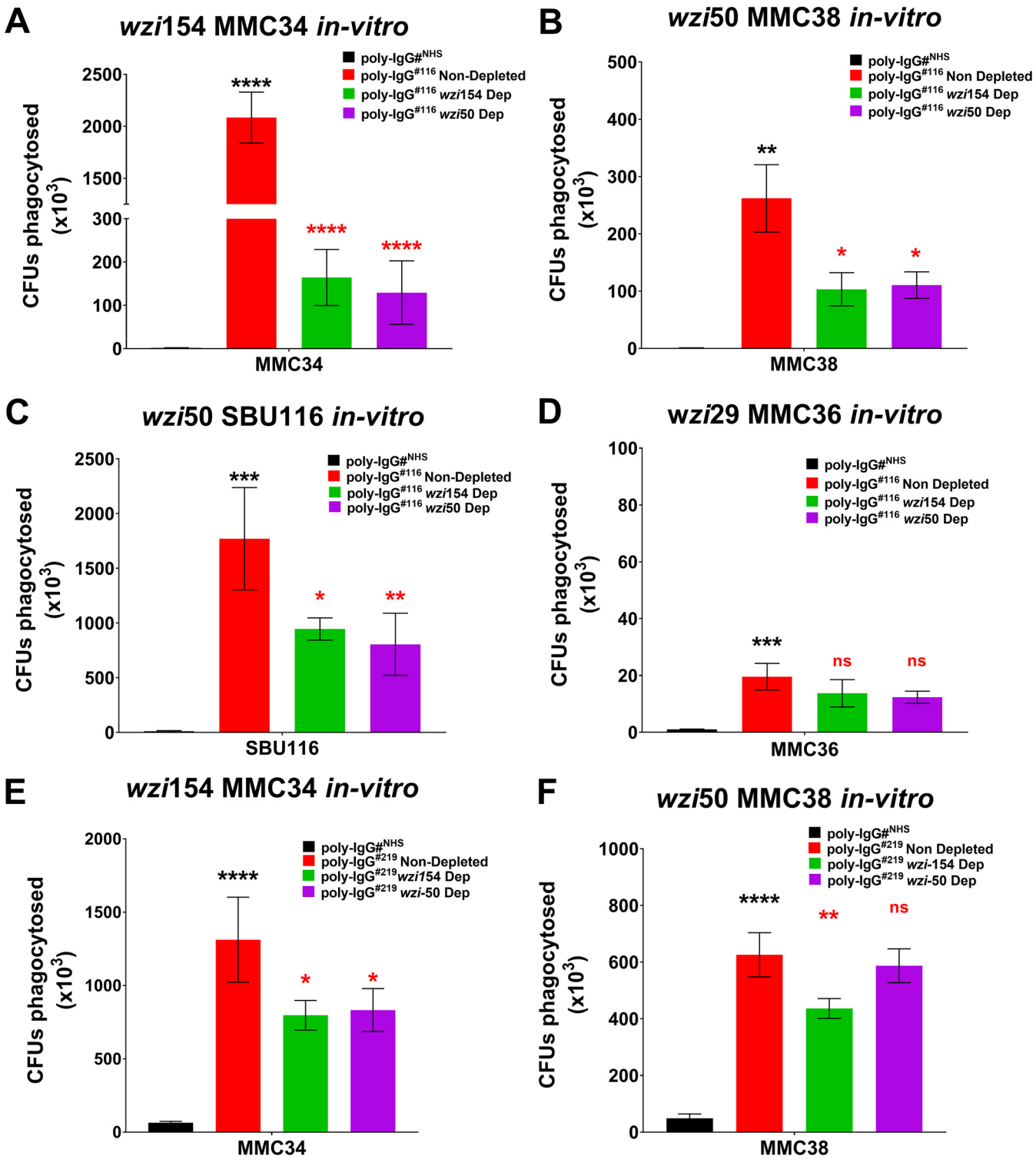

FIG 4 Depletion of CPS-specific antibodies inhibits opsonophagocytosis of corresponding *K. pneumoniae*. The PD IgGs poly-IgG$^{\#116}$ and poly-IgG$^{\#219}$ provided cross-protection against all clade types (including clade 1 and clade 2). Depletion of *wzi154* and *wzi50* CPS-specific IgGs from poly-IgG$^{\#116}$ significantly inhibited opsonophagocytosis of (A) *wzi154* MMC34, (B) corresponding *wzi50* MMC38, and (C) patient-matched SBU116 strains, but (D) the effect on opsonophagocytosis of *wzi29* MMC36 was negligible. Depletion of *wzi154* and *wzi50* CPS-specific IgGs from poly-IgG$^{\#219}$ significantly inhibited opsonophagocytosis of (E) the corresponding *wzi154* MMC34 strain, whereas (F) depletion of *wzi154* CPS only inhibited the opsonophagocytosis of *wzi50* MMC38. Bars depict means and SD from three independent experiments, with wells performed in triplicate. poly-IgG$^{\#NHS}$ serve as the negative controls for the assay. Phagocytosed bacteria within macrophages were counted by plating lysed macrophages (log CFU phagocytosed) on LB agar plates. Overall differences between treatment groups were determined to be significant by repeated-measures two-way ANOVA using Tukey's *post hoc* test, displayed in-graph. For all in-graph statistics, *P* values displayed in black are comparisons to the poly-IgG^{NHS} group, whereas *P* values in red compare nondepleted poly-IgGs with *wzi154*- and *wzi50*-depleted Abs. *P* values are replaced with ns if >0.1, * if <0.05, ** if <0.01, and *** if <0.001.

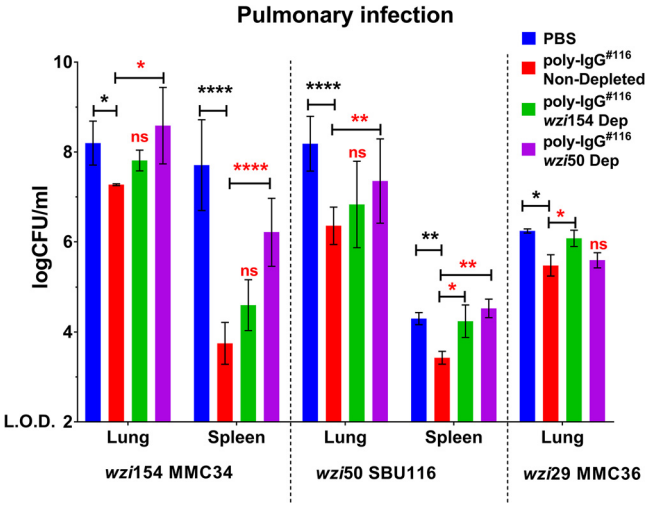

FIG 5 Passive transfer of purified human polyclonal IgG from CR-*Kp*-infected subjects reduces *K. pneumoniae* bacterial burden in CR-*Kp*-infected mice, whereas specific CPS depletion (Dep) reverses the therapeutic effect. Bacterial burden in lungs and spleens of mice infected with a lethal inoculum of MMC34, SBU116, and MMC36 strains preopsonized with either CPS-specific depleted or nondepleted PD IgGs. For all studies, bars depict means, and SD for overall differences in CFU between treatment groups ($n = 3$ per group) were assessed for significance by two-way ANOVA with multiple-comparison correction and the limit of detection (LOD) set at $y = 2$. For all in-graph statistics, *P* values displayed in black are comparisons to the PBS group, whereas *P* values in red compare nondepleted poly-IgGs with *wzi154*- and *wzi50*-depleted Abs. *P* values are replaced with ns if >0.1, * if <0.05, ** if <0.01, and *** if <0.001.

ultimately cleared *Klebsiella*. Instead, we chose to further analyze the Ab response to the three most common *wzi* types of ST258 strains. The *wzi154* CPS type is expressed by most clade 2 strains, *wzi29* is the most prevalent CPS expressed by clade 1 strains, and *wzi50* can be expressed by clade 1 as well as clade 2 strains (7, 8, 29). Interestingly, three of the seven patients who did not mount a CPS-specific Ab response were infected with less common *wzi* types (*wzi173* and *wzi7*).

Despite the CPS variability in CR-*Kp*, plasma from many of the patients reacted to more than one CPS. This was not necessarily expected because the structure of *wzi154* (MMC34) is distinct from other published *K. pneumoniae* CPS structures (30), and its sugar composition is also very different from that of *wzi50* (7). Regardless, about half of the patients infected with *wzi154* CR-*Kp* strains mounted high titers to *wzi154* and *wzi50* CPS. Interestingly, plasma derived from patients infected with *wzi29* CR-*Kp* exhibited the most pronounced cross-reactivity with other *wzi* types, but no reactivity with *wzi29* CPS. Although this result was consistent with published studies reporting failure of *wzi29* CPS to elicit Abs in mice or rabbits (16, 17), it was not in accord with results from *in vitro* phagocytosis assays. They indicated opsonophagocytic efficacy of PD poly-IgG of *wzi29* CR-*Kp* strains, whereas acapsular mutants were not phagocytosed, consistent with the presence of Abs specific for *wzi29*-type CPS. Although these results are not conclusive, a modified ELISA demonstrating Abs to whole *wzi29* CR-*Kp* further supports the conclusion that *wzi29*-type CPS indeed elicits Abs. We hypothesize that critical CPS epitopes are destroyed during CPS purification, and better methods to purify CPS are needed. Cross-reactive Abs have been identified in the serum of volunteers vaccinated with CPS derived from different *K. pneumoniae* strains (31). Such cross-reactivity may be the result of cocktails of distinct Abs that bind to different antigens or, alternatively, some Abs could react with more than one CPS. Indeed, one study reported that 12% of human MAbs cloned from subjects vaccinated with 23-valent Pneumovax cross-reacted with two serotypes (32). Although our murine monoclonal antibodies (MAbs) exclusively bind to *wzi154*-type CPS, one could potentially clone hybridomas that produce cross-reactive Abs through modified vaccination protocols and altered screening procedures.

Importantly, healthy subjects did not exhibit Ab reactivity with *Kp*-derived CPS or LPS, indicating that these Ab reactivities are the result of infection and colonization with CR-*Kp*. Further studies will need to resolve if CPS-specific IgA is present in the colon. Interestingly, the dominance of a specific IgG subclass was not observed for CPS-specific Abs, whereas the LPS of *Klebsiella* elicited predominantly IgG2 response. Although one vaccine study in mice with CR-*Kp* oligosaccharides identified the murine subclass mIgG1 to be the most seroprevalent in those mice (33), human IgG2, which is similar to the analogous murine isotype (mIgG3), is the primary humoral response to T-independent antigens such as CPS. This IgG subclass preference was also observed in humans infected with *Helicobacter pylori* and *Mycobacterium tuberculosis* (34, 35).

Abs directed against the O polysaccharide are protective against wild-type *Kp* in lethal infection models (36, 37). Abs that bind to LPS have not only been shown to be opsonophagocytic but also cross-reactive (22). We do not believe that cross-contamination of CPS with LPS is responsible for the cross-reactivity in our patient plasma because highly sensitive tests were used to rule out LPS contamination during CPS purification, which was done according to standard protocols (see Fig. S5 in the supplemental material). Furthermore, cross-reactivity was not observed with all patient plasma. Last, experiments with acapsular mutants, as well as studies with bulk plasma that was depleted of CPS-specific Abs, were performed to show the causal role of anti-CPS antibodies in opsonophagocytic efficacy and document their respective cross-reactivity. The first set of experiments demonstrate that bulk IgG promotes agglutination of two CR-*Kp* strains (*wzi50* and *wzi154*) and enhances serum resistance in all three *Klebsiella* strains, which differ in agglutination and serum resistance (12, 16, 17). Ab-mediated complement deposition of C5b-9 and C3c has been shown to be critical for Ab activity (16). Enhancement of opsonophagocytic efficacy by two different high-titer IgG bulk fractions demonstrated that enhancement of phagocytic activity was mediated by CPS-specific Abs and lost after depletion. Again, cross-reactivity was observed for MMC34 (*wzi154*), as both depletion of *wzi50* and of *wzi154*-specific Abs affected the phagocytic efficacy.

In vivo efficacy of poly-IgG$^{\#116}$ in a pulmonary infection model further supports our conclusion that infected patients generate a protective humoral immune response and that CPS-specific Abs are relevant Abs in both bulk IgG fractions. PD poly-IgG$^{\#116}$ lowered bacterial burden in the lung and dissemination to the spleen in mice infected with MMC34, MMC36, and MMC38, which expressed *wzi154*-, *wzi29*-, and *wzi50*-type CPS, respectively. Importantly, protective efficacy was reversed when CPS-specific Abs were depleted, underscoring their relevance. Regrettably, CR-*Kp* strains exhibit low virulence, and *in vivo* experiments continue to be a challenge in mice (16, 38) and even in cynomolgus macaques (19). Our laboratory is actively pursuing studies to investigate if anticapsular Abs can also be given prior to infection and prevent disseminated disease. One of the major limitations of the current animal model is that high inocula are required to measure the effect on dissemination. Historically, murine models have used more virulent *Klebsiella* strains, which require several log lower inocula to kill mice. Recent advances with a neutropenic murine model could potentially allow experiments with lower inocula (39).

In summary, despite marked capsular heterogeneity, our results demonstrate that infection with CR-*Kp* induces cross-reactive anti-CPS Abs. The finding that most (79%) elderly patients, irrespective of their state of infection, mounted an Ab response to at least one of the three CPS underscores the immunogenicity of CPS and indicates the feasibility of a CPS-based vaccine strategy that targets the elderly, who are also the most vulnerable. A limitation of the study was that the long-term persistence of IgG was not evaluated. However, limited uncontrolled data from healthy subjects who have IgG to *Klebsiella* CPS indicated that the humoral response persists for months. In contrast to *S. pneumoniae*, CR-*Kp* colonization is not yet widely spread in the community, and the majority of patients still become colonized through nosocomial exposure in diverse health care settings (40, 41). Recent advances with recombinant production

of bioconjugate vaccines in glycoengineered *Escherichia coli* cells against the 2 *K. pneumoniae* serotypes, K1 and K2, could improve the generation of multivalent CPS-based vaccines (25). Our data indicate that vaccines with *wzi154*, *wzi*29, and *wzi50* could potentially give broad coverage, which is preferable because, similarly to pneumococcal vaccines, non-vaccine CPS types could increase in prevalence after vaccine introduction (42, 43). In summary, our results encourage efforts to further develop CPS-based vaccines. Furthermore, these data suggest that certain CPS may elicit more cross-reactive Abs, but care has to be taken when purifying CPS to conserve immunogenic epitopes.

MATERIALS AND METHODS

Ethics statement. Animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University (SBU; approval no. 628253). This study is in strict accordance with federal, state, local, and institutional guidelines that include the Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the Public Health Service Policy on Human Care and Use of Laboratory Animals. All surgery was performed under ketamine-and-xylazine anesthesia, and every effort was made to minimize suffering. Patients consented under institutional review board (IRB)- and SBU Human Subjects Committee-approved protocols (IRB no. 896845 and 851803). Healthy donors gave written informed consent for blood donation under IRB no. 718744.

Collection of plasma and CR-*Kp* from patients. Patients admitted to Stony Brook University Hospital (SBUH) from 2017 to 2019, from whom CR-*Kp* was isolated, consented and their CR-*Kp* strains and plasma were collected and frozen at −80°C and −20°C, respectively. Patients were classified as being "colonized" or "infected" based on standard criteria described elsewhere (11).

CR-*Kp* strain isolation, *wzi* typing, and purification of capsular polysaccharide. CR-*Kp* strains were identified by standardized methods according to the Clinical and Laboratory Standards Institute (CLSI), as well as revised CDC criteria (3), and *wzi* typed according to published protocols (5). For experiments, CR-*Kp* strains isolated from patients, as well as MMC36 (*wzi29*), MMC34 (*wzi154*), and MMC38 (*wzi50*) (7), were cultured in Luria-Bertani (LB) broth and agar at 37°C (7).

The CPS of the three previously characterized strains was purified for ELISA and depletion studies based on a previously described protocol in Supplemental Methods S1 of Diago-Navarro et al. (15). Briefly, overnight cultures in 1 liter of LB broth were pelleted by centrifugation, washed with PBS, and resuspended to 5% (wt/vol) in distilled water. CPS was extracted by phenol-water in the aqueous phase and precipitated by adding 5 volumes of methanol plus 1% (vol/vol) of saturated solution of sodium acetate for 2 h at −20°C. After dissolving the pellet in water and dialyzing it against water, it was lyophilized. Proteins and nucleic acid contaminants were removed by digesting them with proteinase K (50 mg/ml) and nucleases (50 mg/ml of DNase I and RNase A) twice for 24 h. LPS was removed by ultracentrifugation (105,000 × *g*, 16 h, 4°C), and samples were freeze-dried. CPS was further extracted with phenol and was further purified by a size exclusion chromatography on an S-200 HR column (GE Healthcare Life Sciences) with PBS. Fractions were collected, and the presence of polysaccharide was analyzed by the phenol-sulfuric acid method (15).

To ensure that the purified CPS samples were devoid of lipopolysaccharide (LPS), we performed a sodium deoxycholate (DOC)-PAGE analysis that can detect up to 0.5 ng endotoxin per ml of sample. DOC-PAGE analysis was done with both LPS (L4268; Sigma-Aldrich) and purified CPS samples (200 ng and 100 ng). LPS contamination was detected by silver nitrate staining (44). LPS was not detected in the gel for CPS samples (see Fig. S5 in the supplemental material). Absence of LPS (<20 endotoxin units [EU]/ml endotoxin limit for polysaccharide vaccines [45]) in purified CPS was further confirmed by the standard Pierce LAL chromgenic endotoxin kit (Thermo Scientific), which is more sensitive and has a lower detection limit of 0.01 EU/ml (0.01 ng endotoxin per ml) (Fig. S5) (19).

Detection of anti-CPS and anti-LPS antibodies in plasma by CPS or LPS ELISA. ELISAs described previously (15) were employed to detect circulating CPS Abs in the plasma of patients. Plasma samples were added in 1:50 dilution (determined by titration, 1:25 to 1:150) for all ELISAs, and secondary anti-human IgG, IgM, IgA, and IgG subclass antibodies were used for detection. The presence of CPS-specific Abs was defined by an OD ratio value of ≥2 (OD ratio = optical density at 405 nm [OD_{405}] CPS/OD_{405} bovine serum albumin [BSA]), where an OD ratio value of <2 was considered nonspecific. For detection of lipopolysaccharide (LPS)-specific Abs, a similar ELISA was done using *K. pneumoniae* LPS purchased from Sigma-Aldrich (L4268).

Bulk IgG isolation. Bulk patient-derived poly-IgGs (PD IgGs) were purified from plasma of patients infected with SBU116, SBU168, SBU207, or SBU219 or normal human serum (NHS) (H4522; Millipore Sigma) by negative selection using Melon gel resin (Thermo Scientific) according to the manufacturer's protocol. Bulk IgGs were quantified using the Human IgG ELISA development kit (Mabtech).

Bulk IgG serum resistance assay and rapid agglutination assay. A human serum resistance assay was done with poly-$IgG^{\#168}$ (40 μg/ml) by modifying the protocol described in (46). Agglutination assays of MMC34, MMC36, and MMC38 strains were carried out with NHS-derived IgG (poly-IgG^{NHS}) or with PD IgGs isolated from a patient infected with SBU168 (40 μg/ml) as described previously (15, 16). Briefly, 3×10^6 CR-*Kp* strains were incubated with poly-$IgG^{\#168}$ in 75% NHS at 37°C for 0-, 1-, 2-, and 3-h time intervals. At each interval, samples were diluted serially and plated on LB plates. The experiment was repeated thrice.

Agglutination assays of MMC34, MMC36, and MMC38 strains were carried out with NHS-derived IgG (poly-IgG^{NHS}) or with PD IgGs isolated from a patient infected with SBU168 (40 μg/ml) as described previously (15, 16). Agglutination was captured in phase-contrast at a magnification of $\times$200 with a Zeiss deconvolution microscope, and images were analyzed with ImageJ software. The experiment was repeated thrice.

Whole-cell ELISA. A whole-bacterium ELISA (25) was used to detect Abs to *wzi*29-type CPS-expressing MMC36. Briefly, half of the 96-well plates (48 wells) were coated with 8×10^8 CFU $\cdot$ ml^{-1} of *wzi*29 MMC36 strain in methanol for 24 h. After 24 h, the plate was blocked with 2% BSA, and a standard anti-CPS ELISA was performed. Presence of Abs in plasma was defined as an OD ratio value of $\geq$2, and plasma with an OD ratio value of $<$2 was considered to have no/negligible anti-MMC36 antibodies.

Biotinylation of capsular polysaccharides and depletion of CPS-specific IgGs. CPS biotinylation was done by adapting a protocol described previously (47). Briefly, purified CPS was constituted in endotoxin-free water at 1 mg/ml concentration. Two hundred microliters of sodium periodate (100 mM) were added per 1 mg CPS, and the solution was incubated for 6 h. Then, 100 μl of 25% glycerol was added to react with excess sodium periodate, and the solution was left at room temperature in the dark for 2 h. After periodate cleanup, the reaction mixture was passed through two Bio-Gel P-2 minicolumns (catalog no. 1504114; Bio-Rad) by centrifugation (3,000 rpm, 2 min) per the manufacturer's instructions. Biotin-hydrazide (SP-1100; Vector Laboratories) was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/ml. For labeling, 40 μl (2 mg) of this solution was mixed with 1 mM manganese dichloride per 1 ml of CPS solution and incubated overnight at room temperature. Modified polysaccharides were then passed through two Bio-Gel P-2 minicolumns and reduced with 1 mM sodium borohydride for 5 min at room temperature. The reduced polysaccharide was passed through two Bio-Gel P-2 minicolumns, and the level of biotinylation was detected with a Pierce biotin quantitation kit (catalog no. 28055; Thermo Scientific).

Pierce NeutrAvidin agarose beads (200 μl, catalog no. 29200; Thermo Fisher Scientific) were coated with 1 mg/ml biotinylated CPS at 4°C for 16 h. Coated beads were washed with 2 column volumes of PBS (pH 7.4) and then incubated with bulk PD IgGs at 4°C for 16 h. Beads were washed with 2 column length volumes (2 ml) of PBS (Corning), and the CPS-depleted pools were eluted with PBS for further use. Depletion was confirmed by CPS ELISA before use in further experiments.

Macrophage phagocytosis. Macrophage cell line J774A.1 (ATCC) was used for macrophage phagocytosis assays with bulk PD poly-IgGs (40 μg/ml) using published protocols (15, 16). Briefly, 1×10^5/ml J774A.1 cells were incubated overnight in wells of cell culture-treated 96-well plates. The following day, 1×10^6 bacteria/ml were opsonized for 60 min in Dulbecco's modified Eagle's medium (DMEM) containing 40 μg/ml of either poly-IgGs or control poly-IgG^{NHS}, and 100 μl of this (multiplicity of infection [MOI] = 1) was added to each well of the washed macrophage plates. After 30 min of incubation at 37°C in 5% CO_2, cells were washed three times with DMEM alone. Macrophages were then exposed to medium containing 100 μg/ml of polymyxin B for 30 min to kill off bacteria that were not phagocytosed and were present outside the cells. Cells were washed again five times, and wells were immediately lysed twice with water and dilution plated on LB plates. The number of CFU calculated from LB plates was divided by the number of estimated cells plated to give the number of CFU phagocytosed.

Poly-IgG$^{\#168}$, poly-IgG$^{\#116}$, poly-IgG$^{\#207}$, and poly-IgG$^{\#219}$ were used to study antibody-mediated opsonophagocytosis of CR-*Kp* strains (SBU168, SBU116, SBU207, SBU219, MMC34, MMC36, and MMC38) and of *wzi154* CPS-expressing 33576 and the acapsular 33576 Δ*wzy* mutant (13). Phagocytosis assay was additionally carried out with CPS-specific depleted PD IgGs (*wzi50*- and *wzi154*-depleted poly-IgGs) and poly-IgG$^{\#NHS}$ (negative control). All conditions were performed in triplicates.

Pulmonary infection model in mice. The pulmonary infection model study was done per a protocol described previously (15). Female C57BL/6 mice (6 to 8 weeks old) were used. CR-*Kp* strains (MMC36, MMC34, and SBU116; 2×10^8 CFU/ml) were incubated with PD poly-IgGs, CPS-specific depleted PD poly-IgGs, or PBS at 5 mg/ml for 1 h. A 50-μl volume of this inoculum, containing 10^7 CFU, was injected intratracheally. After 24 h, mice were euthanized, and lungs and spleens were processed for enumeration of bacteria in homogenized tissue and bacterial dissemination analysis.

Statistical analysis. Statistical tests were performed with GraphPad Prism 6 for Windows. For multigroup comparisons of parametric data (e.g., phagocytosis and serum resistance assay), analysis of variance (ANOVA) with *post hoc* analysis using Tukey's, Sidak's, or Dunnett's comparison tests was used. For two-group comparisons of parametric data, paired *t* tests corrected for multiple comparisons using the Holm-Sidak method were performed.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.6 MB.

FIG S2, TIF file, 1.4 MB.

FIG S3, TIF file, 0.9 MB.

FIG S4, TIF file, 0.9 MB.

FIG S5, PDF file, 0.1 MB.

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We report no financial conflicts of interest.

K.B., M.P.M, E.D.-N., and B.C.F. contributed equally to the ideation and development of the project. K.B. conducted all of the experiments, analyzed the data, and generated all of the manuscript figures. K.B., M.P.M., and B.C.F. contributed equally to writing and editing the manuscript. E.D.-N. contributed to patient plasma and clinical isolates collection, and *wzi* typing. M.P.M. helped K.B. in conducting animal experiments.

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