Dual-Function Antibodies to *Yersinia pestis* LcrV Required for Pulmonary Clearance of Plague

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*Yersinia pestis* causes pneumatic plague, a necrotic pneumonia that rapidly progresses to death without early treatment. Antibodies to the protective antigen LcrV are thought to neutralize its essential function in the type III secretion system (TTSS) and by themselves are capable of inducing immunity to plague in mouse models. To develop multivalent LcrV antibodies as a therapeutic treatment option, we screened for monoclonal antibodies (MAbs) to LcrV that could prevent its function in the TTSS. Although we were able to identify single and combination MAbs that provided the high-level inhibition of the TTSS, these did not promote phagocytosis in vitro and were only weakly protective in a mouse pneumonic plague model. Only one MAb, BA5, was able to protect mice from pneumonic plague. In vitro, MAH BA5 blocked the TTSS with efficiency equal to or even less than that of other MAbs as single agents or as combinations, but its activity led to increased phagocytic uptake. Polyclonal anti-LcrV was superior to BA5 in promoting phagocytosis and also was more efficient in protecting mice from pneumonic plague. Taken together, the data support a hypothesis whereby the pulmonary clearance of *Y. pestis* by antibodies requires both the neutralization of the TTSS and the simultaneous stimulation of innate signaling pathways used by phagocytic cells to destroy pathogens.

*Yersinia pestis*, the etiologic agent of bubonic, pneumonic, and septicemic plague, has been responsible for more human death than any other bacterial pathogen (42). Fortunately, naturally occurring cases of plague in humans now are uncommon, largely due to advances in basic sanitation and public awareness of infectious disease (32). Nevertheless, the disease remains endemic in many areas of the world, and periodic outbreaks continue to occur each year. *Yersinia pestis* is believed to have evolved recently from *Y. pseudotuberculosis*, acquiring flea transmission and respiratory invasion properties through mobile genetic elements (1, 9). The flea transmission cycle provides an opportunity for further evolution, because the bacteria reside in the nonsterile environment of the flea gut, where the formation of a biofilm provides an opportunity for horizontal gene exchange with other microbes (30). Multidrug-resistant *Y. pestis* isolates have been recovered from human plague patients, suggesting that the bacteria do indeed continue to evolve mechanisms of survival in the mammalian host (22, 25, 54). For these reasons, as well as for its potential use as a biological weapon, *Y. pestis* continues to be a significant public health concern and is a priority pathogen for the development of new vaccines and alternative therapeutics (32, 43).

There currently are no plague vaccines that are licensed for human use in the United States. The licensing of current candidates is likely to fall under the U.S. Food and Drug Administration’s Animal Rule for the demonstration of efficacy and potency due to a lack of naturally occurring human plague cases (19). Thus, efficacy trials and the evaluation of vaccine potency in humans will be dependent on our ability to understand the molecular mechanism of protection. Current subunit vaccine candidates are formulated from two protective antigens, Fraction 1 (F1) and LcrV, which are undergoing extensive testing to satisfy the Animal Rule requirements (2, 5, 13, 26, 55, 57–59). Both antigens elicit a neutralizing antibody response that can be translated to passive antibody or even gene therapies (2, 4, 13, 28, 37, 48). These protective antibodies act directly on the bacteria and alter its interactions with innate immune cells such that the host clears the infection. T-cell responses also are believed to play an important role in host defense against *Yersinia pestis* (40, 41).

CaF1, or F1, is an abundant cell surface antigen of the type I pilin family that forms a capsule-like structure on *Y. pestis* at 37°C (8). Although F1 appears to be antiphagocytic, it is not essential for virulence and thus would not contribute to immunity against *Y. pestis* mutant *caF1* (18, 21). In contrast, LcrV is essential for all forms of plague due to its role in the type III secretion system (TTSS) (12, 45, 47). LcrV is positioned on the surface of bacteria at 37°C, where it mediates the translocation of anti-host factors, collectively known as *Yersinia* outer proteins (Yops), whose antiphagocytic, cytolytic, and proapoptotic activities allow *Yersinia* to avoid being killed by the host’s immune system (38, 46). Polyclonal antibodies to recombinant LcrV (α-LcrV) can bind to this needle tip and lead to the inhibition of the TTSS and the phagocytosis of the bacteria (14, 24, 53). However, it remains controversial whether the direct inhibition of the TTSS by α-LcrV leads to phagocytosis or if the direct promotion of phagocytosis leads to the inhibition of the TTSS because it cannot function intracellularly (59, 60). Three monoclonal antibodies (MAbs) have been independently cloned that can protect mice from bubonic and pneumonic plague (2, 27, 48). Although it is unclear whether each of these targets the same epitope, deletion studies of LcrV antigen suggest multiple protective epitopes exist (13, 39, 44, 51).

We were interested in developing antibody therapeutics and...
maximizing the potency of anti-LcrV therapy. In this work, we investigated the mechanism of protection from pneumonic plague to determine if the multivalent occupancy of antibody to LcrV improved protection. We found that antibodies that promoted phagocytosis directly were more potent at neutralizing pneumonic plague, although the inhibition of the TTSS alone led to partial protection. Only a single LcrV epitope led to antibodies that by themselves promoted uptake, while the multivalent occupation of antigen with MAbs did not increase either phagocytosis or protection. These data provide new insight into the mechanism of LcrV and support the use of assays that measure the phagocytic uptake of Y. pestis as correlates of immunity for the evaluation of plague vaccines.

MATERIALS AND METHODS

Bacterial strains. All Y. pestis strains used were taken from frozen stocks and streaked for isolation onto heart infusion agar (HIA) plates. For pneumonic plague vaccine, Y. pestis CO92 was plated on HIA supplemented with 0.005% Congo Red and 0.2% galactose to verify the presence of the pigmentation locus (49a). Pigmented, isolated colonies then were inoculated in heart infusion broth (HIB) supplemented with 2.5 mM CaCl2 and grown for 18 to 24 h at 37°C, followed by dilution to the desired dose in sterile phosphate-buffered saline (PBS). All experiments with Y. pestis CO92 were performed in compliance with select-agent regulations and in accordance with the guidelines outlined by the University of Missouri Institutional Biosafety Committee. For in vitro assays with macroporous, Y. pestis KIM D27, a nonpigmented strain originally isolated by R. Brubaker (8a), was grown routinely fresh from frozen stock on HIA, followed by aerobic growth at 27°C in HIB overnight prior to use in experiments. An isogenic derivative of KIM D27 lacking the 70-kb virulence plasmid that encodes the aerobic growth at 27°C in HIB was used to induce the suicide vector pCVD42 into pCD1, followed by selection for the loss of both; the resulting mutant strain was confirmed by PCR analysis and Western blotting (17). The Escherichia coli strain JM109 (a gift from George Stewart) or DH5α was used routinely for cloning expression plasmids; E. coli BL21 (Novagen, Madison, WI) was used for protein purification. Ampicillin (Amp) was used at 100 μg/ml for experiments involving recombinant plasmids.

Plasmids. pNE071 expresses DsRed from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter on a pUC18 plasmid backbone. The DsRed gene was amplified from the pDsRed-Monomer vector (Clontech, Mountain View, CA) with primers that included Ndel and BamHI sites for cloning. The lacI gene and downstream tac promoter originally were amplified from pGEX-2TK (GE Healthcare, Buckinghamshire, United Kingdom) (3) with abutted EcoRI and NdeI restriction sites, followed by being cloned into pDsRed Monomer vector such that these sequences replaced the endogenous promoter. Production of recombinant. Recombinant LcrV was overexpressed in E. coli BL21 and purified as previously described (39). LcrV then was used as an antigen for the wiffle ball immunization of New Zealand White rabbits (11). All experiments employing polyclonal anti-LcrV came from the same immunized animal. Rabbit serum containing anti-LcrV antibodies was applied to a Protein A column and purified by following the manufacturer’s protocol (Sigma, St. Louis, MO). Samples then were applied to a PD-10 desalting column (GE Healthcare, Buckinghamshire, United Kingdom) and eluted in PBS. Total immunoglobulin G (IgG) was quantified using bovine IgG as a standard in a bicinchoninic acid protein assay (Pierce, Rockford, IL).

Cloning of LcrV MAb E11. All monoclonal antibody (MAb) clones except E11 were generated against recombinant LcrV in immunized BALB/c mice and will be described elsewhere (O. Schnewind, personal communication). For the cloning of MAb E11, amino acids 241 to 270 of LcrV were chemically synthesized as a peptide conjugated to keyhole limpet hemocyanin (Biothesis, Inc., Lewisville, TX). This peptide conjugate was used by the University of Chicago Fitch Monoclonal Antibody Facility to immunize BALB/c mice for cloning antibodies with specificity to rLcrV. Positive clones were selected by screening for binding to rLcrV by enzyme-linked immunosorbent assay (ELISA), and the MAb (E11) with greatest relative affinity for rLcrV was isolated and selected for analysis.

Purification of MAbs. MAbs were produced and purified according to standard methods by the University of Chicago Fitch Monoclonal Antibody Facility. Briefly, B-cell hybridomas expressing monoclonal antibodies were grown either in culture or in a bioreactor in serum-free medium. Antibody was harvested from the culture supernatants and purified using Protein G affinity chromatography. MABs were eluted in 0.1 M glycine hydrochloride, pH 2.6, and dialyzed in PBS for 24 h with two buffer exchanges. The MAb concentration was determined by Bradford assay using bovine IgG for a standard curve. MAbs were stored at -80°C.

ELISA. LcrV was used as the capture antigen for both ELISA and competitive ELISA experiments. Ninety-six-well plates were coated with 100 μg rLcrV and blocked with 1% bovine serum albumin (BSA) in wash buffer (0.01% Tween in PBS). Wells then were probed with LcrV MAb for 2 h, followed by detection with phosphatase-labeled goat anti-mouse Ig antibody. Error bars represent the standard deviations from three separate assays.

Purification of MAbs. MAbs were produced and purified according to standard methods by the University of Missouri Institutional Biosafety Committee. For in vitro assays with macroporous, Y. pestis KIM D27, a nonpigmented strain originally isolated by R. Brubaker (8a), was grown routinely fresh from frozen stock on HIA, followed by aerobic growth at 27°C in HIB overnight prior to use in experiments. An isogenic derivative of KIM D27 lacking the 70-kb virulence plasmid that encodes the
co2. At this time, gentamicin (100 μg/ml) was added to macrophages in a final volume of 2 ml, spun 

10/min at 37°C. Suspensions were applied to macrophages in a final volume of 600 μl, and then incubated for 2 h. Cells were incubated for an additional 2 h. Cells then were fixed with 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) and streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Z-stacked images were acquired on an Olympus IX70 inverted widefield fluorescent microscope from at least four random fields of 50 macrophages, and bacteria were counted in single-blind fashion. Representative images were acquired using Zeiss LSM 510 META NLO confocal laser-scanning microscopy.

**Animals.** Six- to 8-week-old female C57BL/6 mice (16 to 20 g; Charles River Laboratories, Wilmington, MA) were used for plague challenge experiments. During challenge, mice were maintained in select-agent-approved containment facilities at the University of Missouri in accordance to the guidelines outlined by the institutional animal care and use committee. All infected mice were monitored regularly by daily weighing and the assignment of health scores. Animals were euthanized by CO2 asphyxiation, followed by cervical dislocation, methods that are approved by the American Veterinary Medical Association guidelines on euthanasia.

**Pneumonic plague challenge.** Bacteria grown as described above at 37°C were diluted in sterile PBS to 6,000 CFU/0.02 ml, which corresponds to 20 50% lethal doses (LD50) (15, 35, 52), just prior to use in challenge experiments. Groups of five mice were given antibody (400 μg/0.4 ml) or PBS by intraperitoneal injection 60 min prior to challenge. All animals were lightly anesthetized by isoflurane inhalation prior to intranasal infection with Y. pestis CO92. Animals were observed for recovery from anesthesia and returned to housing.

**Statistical analysis.** One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of caspase-3 data. Dunnnett’s test was used after ANOVA to account for type I errors, and multiple comparisons and the reported P values are the combined result of both tests. The unpaired Student’s t test was used to evaluate the statistical significance of data collected from gentamicin protection assays. The log-rank test was used to evaluate the statistical significance of survival and mean time to death (MTTD).

**RESULTS**

**Characterization of antibodies that neutralize Yersinia pestis.** We recently generated a library of MAbs following the vaccination of BALB/c mice with rLcrV. Within this library, six linear peptide epitopes were represented, with several others binding what appears to be one or more conformational epitopes (O. Schneewind, personal communication). In addition, we cloned a seventh MAb (E11) against a peptide of LcrV amino acids 241 to 270 conjugated to Keyhole limpet hemocyanin. This epitope was not represented in our original library, yet deletion studies suggested that this is a neutralizing epitope (39). We were interested in developing a multivalent LcrV MAb therapy as a postexposure treatment option and sought to identify MAbs that neutralized LcrV function. Representative MAbs from each of the seven epitopes, all of which were of the IgG1 isotype, were selected and measured for relative affinity to purified LcrV in an ELISA. Initial tests to determine binding titers between MAb AH1 and BA5 and rLcrV were performed, and 7.5 μg/ml antibody reproducibly resulted in binding at the peak of the titer curve (data not shown). Therefore, this amount of MAb was chosen to characterize the relative binding of all of the MAbs with antigen rLcrV. Although there were various degrees of binding between MAb and

![FIG. 2. MAbs BA5 and AH1 block injection of YopJ into macrophages. One hundred micrograms of antibody or an equivalent volume of PBS was preincubated with Y. pestis KIM D27 at 37°C for 1 h before infecting RAW 264.7 macrophage-like cells. pCD1 Y. pestis KIM D27 treated with PBS was used as a control for no injection. Pretreated bacteria then were added to macrophages at an MOI of 10. Caspase-3 activation was measured 3.5 h after infection. Error bars represent the standard deviations from the means from at least six independent experiments. Statistical significance compared to results for untreated KIM D27 was evaluated by one-way ANOVA. *, P < 0.05.](http://cvi.asm.org/)

![FIG. 3. Multivalent antibody binding to LcrV results in the increased blocking of the TTSS. MAbs that were mixed in equal amounts, totaling 100 μg antibody, or an equivalent volume of PBS was preincubated with Y. pestis KIM D27 at 37°C for 1 h before infecting RAW 264.7 macrophage-like cells at an MOI of 20. The infection of macrophages with pCD1 Y. pestis KIM D27 treated with PBS was used as a control for no injection. Caspase-3 activation was measured 3.5 h after infection. Error bars represent the standard deviations from the means from at least three independent experiments. Statistical significance compared to results for untreated KIM D27 was evaluated by one-way ANOVA. *, P < 0.05.](http://cvi.asm.org/)
antigen, all MAbs had detectable binding to LcrV at 7.5 μg/ml (Fig. 1).

Single MAbs initially were characterized for their ability to block the type III injection of Yops in vitro. For this analysis, we modified a caspase-3 assay that currently is used to evaluate the potency of LcrV vaccines (7, 53). Wild-type Y. pestis KIM D27 injects effector Yops, one of which, YopJ, causes the activation of caspase-3 and the apoptosis of macrophages (36). Isogenic Y. pestis lacking pCD1, which encodes the TTSS, are unable to activate caspase-3. For each experiment, both wild-type and pCD1 Y. pestis KIM D27 strains were included and set to 100 and 0% caspase-3 activation, respectively. Initial tests were performed to monitor the concentration-dependent increase in antibody activity in this assay using 10 to 200 μg of purified polyclonal and monoclonal anti-LcrV. Results showed peak neutralization activity at 100 μg of antibody, and this amount was used to collect the data described below (data not shown). Preincubation with 100 μg of rabbit polyclonal α-LcrV blocked Yop injection, as only 27% caspase activation was observed (P < 0.05 compared to results for the untreated wild-type infection of macrophages) (Fig. 2). However, two MAbs, BA5 and AH1, were able to block injection, averaging 68 (P < 0.05) and 61% (P < 0.05), respectively, of the levels of the untreated control. Reducing the amount of BA5 or AH1 by half (50 μg) also reduced the inhibition of caspase activation, indicating that these MAbs blocked the TTSS in a concentration-dependent manner (data not shown). All other MAbs were unable to block the TTSS.

Identification of LcrV MAb combinations that exhibit increased activity against the TTSS. The multivalent occupancy of antigen by antibodies may lead to improved or even synergistic neutralizing activity. We tested this first by predicting which combinations of MAbs were likely to be capable of occupying LcrV simultaneously. For this, we used VMD 1.8.6 software (31) and published the structural information of LcrV (16). This approach predicted that both BA5 and AH1 could bind antigen simultaneously. MAb E11 was predicted to bind an epitope that overlapped BA5, thus these two MAbs likely are unable to bind LcrV together. Both MAbs AG7 and 4G2 were predicted to bind antigen when both BA5 and AH1 were occupying LcrV. MAbs BD5 and C12 were predicted to be unable to occupy antigen with BA5 and AH1.

We tested combinations of MAbs, present in equal amounts that added up to 100 μg, in the caspase assay, and the results largely supported the structural predictions. Combining BA5 and AH1 led to better inhibition of caspase than using either MAb alone, with 51% blocking (P < 0.05) compared to that of the untreated control (Fig. 3). In contrast, E11 and BA5 did not improve the blocking of the TTSS compared to that of untreated bacteria (78%, P > 0.05), suggesting that these MAbs indeed are unable to bind antigen together. Double and triple combinations involving MAbs BA5 and/or AH1 were tested, and the combination of BA5, AH1, and 4G2 was selected because it appeared to reproducibly provide the greatest increase in the neutralization of LcrV function (55% caspase activation; P < 0.05).

We confirmed that BA5, AH1, and 4G2 could bind LcrV together in a competitive ELISA. When BA5 was biotinylated, unlabeled BA5 could compete for the binding of rLcrV, but neither AH1 nor 4G2 was able to compete (Fig. 4A). Likewise, when AH1 was biotinylated, unlabeled AH1, but not BA5 or 4G2, was able to compete for the binding of rLcrV (Fig. 4B). Equal amounts of these MAbs were tested by ELISA at 0.1 μg/ml total antibody, and we found that each double combi-
nation, as well as the triple combination, appeared to bind antigen with similar affinity (Fig. 4C). Taken together, these data indicate that MAB combinations that simultaneously bind LcrV result in the improved blocking of Yop injection compared to that of the single-MAB occupancy of antigen.

**Multivalent MAB occupancy of LcrV does not lead to improved protection from pneumonic plague.** To compare the efficacy of individual MABs to those of combinations of MABs in vivo, a pulmonary model of infection was used. In this model, C57BL/6 mice were given 400 µg total antibody by intraperitoneal injection 60 min prior to intranasal infection with 20 LD₅₀ (6,000 CFU) Y. pestis CO92, and survival was monitored for a 14-day period. Data shown were generated in two independent challenges, with five mice per treatment group (n = 10 total) with the exception of 4G2, which was tested in only one experiment (n = 5 for 4G2). Statistical significance was evaluated by the log-rank test.

When 200 µg each of BA5 and AH1 was used as a therapeutic, instead of enhancing the neutralization of bacteria, as would be expected based on the caspase data, reduced protection was seen. Likewise, combining BA5 with 4G2 or with both AH1 and 4G2 resulted in reduced protection. Interestingly, an AH1 and 4G2 combination appeared to lead to partial protection (40%), although this protection was not statistically significant (P > 0.05 compared to results for untreated controls). Thus, the single-dose administration of BA5 alone was the most potent therapy. This result is contrary to the data obtained from the caspase-3 assay, where AH1 was equal to BA5 in potency and combinations of these MABs were superior. We therefore decided to investigate the potency of these antibodies in promoting phagocytosis directly to determine if this correlated with the protection data.

**Antibody protection of pneumonic plague correlates with opsonophagocytosis of bacteria.** Previous work on the mechanism of anti-LcrV on Y. pestis virulence has demonstrated that polyclonal antibodies may both neutralize the function of the

![Graph](https://cvi.asm.org/)

**FIG. 5.** Multivalent occupancy of LcrV does not increase protection from pulmonary challenge by *Yersinia pestis*. C57BL/6 mice were given 400 µg total antibody by intraperitoneal injection 60 min prior to intranasal infection with 20 LD₅₀ (6,000 CFU) *Y. pestis* CO92, and survival was monitored for a 14-day period. Data shown were generated in two independent challenges, with five mice per treatment group (n = 10 total) with the exception of 4G2, which was tested in only one experiment (n = 5 for 4G2). Statistical significance was evaluated by the log-rank test.

### TABLE 1. Summary of in vitro and in vivo activities of LcrV antibodies

| Treatment | % Survivala (no. survived/total) | MTTD | % Caspase-3 activationb | Phagocytosis indexc |
|-----------|-------------------------------|------|------------------------|-------------------|
| PBS       | 20 (4/20)                      | 5.1  | 100                    | 0.95              |
| Polyclonal| 100 (10/10)b                   | NAf  | 26.6 ± 15.2d          | 2.92d            |
| BA5       | 90 (9/10)b                     | 9.0  | 67.5 ± 13.6e          | 2.04f            |
| AH1       | 30 (3/10)                      | 5.0  | 61.4 ± 9.0d           | 1.12             |
| 4G2       | 0 (0/5)                       | 4.6  | 87.2 ± 10.6          | 0.74             |
| BA5 + AH1 | 30 (3/10)                      | 5.6  | 50.6 ± 9.6           | 1.39             |
| BA5 + 4G2 | 10 (1/10)                      | 5.8  | 63.4 ± 12.0          | 1.50             |
| AH1 + 4G2 | 40 (4/10)                      | 6.2  | 61.5 ± 17.2          | 1.21             |
| BA5 + AH1 + 4G2 | 30 (3/10)               | 6.1  | 55.8 ± 12.7         | 1.38             |

| a | Protection from intranasal challenge with 20 LD₅₀ *Y. pestis* CO92, collected from two independent trials. |
| b | P < 0.05 compared to results for PBS-treated controls as determined by log-rank test. |
| c | Percentage of untreated bacteria from an average of four independent experiments with samples run in duplicate. |
| d | P < 0.05 compared to results for PBS-treated controls as determined by one-way ANOVA. |
| e | Data collected from two independent experiments counting four fields of 50 macrophages in each experiment. |
| f | NA, not applicable. |
TTSS and directly stimulate phagocytosis (14, 24, 53). We therefore decided to quantify the ability of LcrV MAbs to stimulate the phagocytosis of bacteria by macrophages and to determine if this activity correlates with the protection we observed in vivo. *Y. pestis* carrying a plasmid expressing DsRed under an IPTG-inducible promoter was used in a microscopy-based gentamicin protection assay (61). Briefly, bacteria were preincubated with PBS or anti-LcrV at 37°C prior to infecting RAW 264.7 macrophage-like cells. Phagocytosis proceeded for 30 min, and then gentamicin was added to kill any remaining extracellular bacteria. When only intracellular bacteria remained, IPTG was added to induce DsRed expression. The average number of intracellular bacteria, identified by red fluorescence, was determined after visualization by microscopy (Fig. 6E). These data were collected from at least three independent experiments, each of which was counted blindly, with 50 macrophages counted in four sections of each slide. The phagocytosis index (PI) was calculated as the number of intracellular bacteria divided by the number of macrophages. Untreated wild-type KIM D27 bacteria often were found within macrophages, and a calculated PI of 1.0 was observed (Fig. 6A and E). In contrast, most of the macrophages infected with *Y. pestis* pCD1− carried multiple bacteria, and a PI of 3.9 was observed (Fig. 6B). These data agree with previously published results and show that bacteria that are unable to perform type III secretion are readily phagocytosed by macrophages (10).

Wild-type bacteria coinoculated with LcrV polyclonal antibodies resulted in increased uptake compared to that of untreated bacteria, with a PI of 2.9 (Fig. 6C). MAb BA5 also was able to promote uptake compared to that of the untreated control, with an average PI of 2.0 (P = 0.024) (Fig. 6D), while MAb AH1 was unable to promote phagocytosis more than the untreated control (PI of 1.1; P = 0.609), even though it is able to block the TTSS. Combinations of MAb BA5 with either AH1 or 4G2 reduced the PI from that of MAb BA5 alone, suggesting that reducing the amount of BA5 used in the assay reduced phagocytosis, and this could not be rescued by the multivalent binding of the other MAbs. Compared to the in vivo challenge results, MAb activity in the phagocytosis assay closely matches observed activity during the pulmonary infection of mice with fully virulent *Y. pestis*. Taken together, these data suggest that neutralizing LcrV antibodies not only inhibit the TTSS but also directly promote phagocytosis, and both activities are required for protection from pneumonic plague.

**DISCUSSION**

Vaccines formulated with F1 and LcrV appear to be highly effective in preventing plague. However, there is concern that their use by American civilians for biodefense may not be necessary or desired by the public, whose current concerns regarding vaccine safety may override their risk of exposure to *Y. pestis*. Because the antibody response strongly correlates with immunity to plague, we have been investigating defined antibody therapeutics as alternatives to vaccination. In this work, we studied defined, multivalent antibodies to LcrV with strong neutralizing activity in vitro as a preventive treatment for pneumonic plague in mice. Although LcrV MAb combinations could increase the neutralization of the TTSS, these MAbs by and large did not improve protection. Protective MAb BA5 exhibited biological activity similar to that of polyclonal anti-LcrV, and it was the only MAb treatment able to promote phagocytosis. Thus, it appears that antibodies must block the TTSS and stimulate phagocytic uptake to prevent rapid bacterial growth in the lung. Moreover, combinatorial MAb therapy targeting multiple defined cell surface antigens or elements of the TTSS that enhance either phagocytic uptake or the inhibition of the TTSS may prove potent against pulmonary *Y. pestis* infections (20, 23, 33).

We described, for the first time, antibodies that could block the TTSS without promoting phagocytic uptake, allowing us to make direct comparisons of the contributions of these distinct

**FIG. 6.** Antibodies that protect against pneumonic plague effectively opsonize bacteria. IPTG-inducible DsRed-expressing KIM D27 and KIM D27/pCD1− strains were incubated with 100 μg total antibody for 1 h and applied to RAW 264.7 macrophage-like cells at an MOI of 20. After 30 min of phagocytosis, gentamicin was added to kill extracellular bacteria, and IPTG was applied to samples for 2 h. Cells then were fixed and examined using confocal laser-scanning microscopy for representative images (A to D) or Z-stacking widefield fluorescent microscopy for quantification (E). Bacteria were counted in 50 macrophages from four random fields, and data were collected from at least three independent experiments. Statistical significance was evaluated using the unpaired Student’s t test. BA5 and polyclonal anti-LcrV treatment resulted in statistically significant increases in phagocytosis compared to that of PBS-treated controls (*, P < 0.05), while all other MAb treatments were not statistically different from PBS-treated controls.
functions. Although it remains difficult to determine whether BA5 activity in the TTSS assay is directly or indirectly caused by phagocytosis, it is likely that the binding of this antibody to the needle tip does inhibit the TTSS. Interestingly, protective LcrV MAbs 7.3 was shown to effectively block the TTSS, which was believed to have an indirect impact on phagocytosis (27, 53, 59). Recently, LcrV amino acid 255 was shown to be critical for MAb 7.3 binding, suggesting that MAb 7.3 and BA5 bind a similar epitope, while AH1 is distinct (29). The comparison of antigen binding properties and different antibody isotypes might yield information about the true number of neutralizing epitopes as well as methods and formulations that can enhance their activity. It is conceivable that only a single LcrV epitope generates antibodies that both block the TTSS and stimulate phagocytosis and, therefore, are protective. The concentration of these antibodies in immune sera generally indicates protective immunity; however, the multivalent occupancy achieved by polyclonal antibody binding, which was highly active in both in vitro assays, may be protective without the development of high-titer antibody to the neutralizing epitope(s). Additional analyses comparing antigen binding between protective LcrV MAbs as well as their in vitro activities ultimately might lead to the development of ELISA-based methods for correlates of protection.

Our results support the hypothesis that the antibody clearance of Y. pestis during respiratory infections is dependent on the activation of phagocytic cells such as macrophages and neutrophils, which perform the major defense against the acute infection of the mammalian lung (6, 49, 50, 56). Recently, it was shown that although gamma interferon and tumor necrosis factor alpha are important systemic host responses to plague, they are dispensable for antibody-induced immunity. This is a surprising result, because these cytokines broadly stimulate both innate and adaptive immune cells (34). Future experiments will aim to understand key signaling pathways for the successful host defense of the mammalian lung against virulent Yersinia pestis.

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