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Bactericidal Monoclonal Antibodies Specific to the Lipopolysaccharide O Antigen from Multidrug-Resistant *Escherichia coli* Clone ST131-O25b:H4 Elicit Protection in Mice

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The *Escherichia coli* sequence type 131 (ST131)-O25b:H4 clone has spread worldwide and become responsible for a significant proportion of multidrug-resistant extraintestinal infections. We generated humanized monoclonal antibodies (MAbs) that target the lipopolysaccharide O25b antigen conserved within this lineage. These MAbs bound to the surface of live bacterial cells irrespective of the capsular type expressed. In a serum bactericidal assay *in vitro*, MAbs induced >95% bacterial killing in the presence of human serum as the complement source. Protective efficacy at low antibody doses was observed in a murine model of bacteremia. The mode of action *in vivo* was investigated by using aglycosylated derivatives of the protective MAbs. The significant binding to live *E. coli* cells and the *in vitro* and *in vivo* efficacy were corroborated in assays using bacteria grown in human serum to mimic relevant clinical conditions. Given the dry pipeline of novel antibiotics against multidrug-resistant Gram-negative pathogens, passive immunization with bactericidal antibodies offers a therapeutic alternative to control infections caused by *E. coli* ST131-O25b:H4.

Escherichia coli is a member of the intestinal commensal flora. Certain variants (pathotypes) of the species, however, can cause either intestinal or extraintestinal infections, such as urinary tract infection, meningitis, or bacteremia (1). Extraintestinal pathogenic *E. coli* (ExPEC) strains harbor a large array of virulence traits that enable them to cause disease outside the intestinal tract. ExPEC strains have been evolving antibiotic resistance, often a combined resistance against most of the clinically relevant antibiotics, such as fluoroquinolones, aminoglycosides, and β-lactam antibiotics. Typically, multidrug-resistant (MDR) strains are compromised in their fitness and virulence, which restricts their prevalence to a nosocomial setting and conversely limits their spread in the community. Some successful MDR clonal lineages do, however, retain high virulence potential (2, 3). The *E. coli* clonal lineage sequence type 131 (ST131)-O25b:H4, first described in 2008 (4, 5), has spread globally not only in hospitals (as do most other MDR clones) but also in the community (6–9). This clone is responsible for ~15% (up to 25% [10, 11]) of all extraintestinal *E. coli* infections and represents the majority of fluoroquinolone-resistant isolates (12) and about half of the extended-spectrum β-lactamase (ESBL)-producing isolates (13). The progressive acquisition of additional resistance phenotypes in ST131-O25b:H4 strains leaves very few effective antibiotics for treatment of patients infected by members of this lineage (14). Even more alarming is the recent appearance of carbapenem-resistant ST131 isolates (15–17). Recently, ST131-O25b:H4 strains were shown to predominate among carbapenem-resistant *E. coli* isolates (18). A major clinical concern is the lack of development of novel antibiotics against Gram-negative pathogens, again leaving very limited treatment options (19). The potential emergence and subsequent spread of pan-resistant *E. coli* strains emphasizes the urgent need to develop alternative therapeutic approaches, such as monoclonal antibodies (MAbs).

Lipopolysaccharide (LPS) of Gram-negative bacteria has long been considered an attractive target for active and passive immunization approaches (20, 21). Antibodies against the lipid A (endotoxin) or core oligosaccharide portions of the LPS molecule are expected to have primarily an antiendotoxin function by neutralizing or sequestering endotoxin in the circulation (20). Their antibacterial effect is restricted because of the low accessibility of these epitopes on live bacteria, as they are masked by the abundant O side chains and/or the capsular polysaccharide (22). Conversely, it has been shown that antibodies specific to the O antigens of LPS can trigger bacterial killing by the complement system alone or, alternatively, through opsonophagocytic killing. In models of bacteremia using different animal species, antibacterial O-specific MAbs afford higher protection than those that target the core oligosaccharide portions of the LPS (23, 24). Bactericidal antibodies directed against the O antigens of LPS may therefore offer an effective therapeutic alternative to antibiotics in the fight against MDR clones. In this article, we describe humanized IgG1 MAbs specific to the conserved O antigen of the *E. coli* ST131-O25b:H4 clone that induce complement-mediated killing *in vitro* and give high protective efficacy in a murine model of bacteremia.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Two previously described ST131-O25b clinical isolates (81009 and 3O) (25, 26) that were confirmed genotypically (MLST typed by the Achtman scheme [27] and O25b-specific PCR) and phenotypically (serotyped by O25 rabbit serum and with O25b-specific MAbs) were used in this study. Strain 81009 expresses a K5-type capsular polysaccharide, while strain 3O expresses a non-K5 capsule, confirmed by the use of a K5-specific lytic phage (Statens Serum Institute). A collection of ST131 strains representing different pulsotypes was kindly provided by G. Peirano and J. Pitout (University of Calgary, Canada) (28).

Bacteria were routinely grown in Luria-Bertani (LB) broth (Fisher Scientific) or on Trypticase soy agar (TSA) plates (bioMérieux).

When bacteria were cultured in the presence of human serum, the serum samples obtained from healthy volunteers were pooled (from a minimum of 3 donors) and depleted of *E. coli*-specific antibodies according to a previously published method (29). Complement was heat inactivated at 56°C for 40 min, and human serum samples were diluted to 50% with 3% human albumin (Albiomin, Biotest), 1.67 diluted RPMI 1640 (Life Technologies), and 12 μM L-glutamine (Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium (Life Technologies).

For *in vivo* experiments, bacteria were grown in LB broth or in pooled human serum (PAA) that was heat inactivated and diluted in RPMI 1640 without phenol red or L-glutamine (Life Technologies) to 50% final concentration.

Generation and selection of humanized monoclonal antibodies targeting the LPS O25b antigen. BALB/cJRj mice were immunized three times with ~1×10^7 CFU of either *E. coli* 81009 or *E. coli* 81009Δ*kps* (30). Mice with the highest titers against O25b antigen were boosted, and their splenocytes were subjected to hybridoma fusion as previously described (30). From five independent fusions, a total of 23 hybridoma clones were selected based on specific binding to purified O25b LPS, assessed by immunoblots and ELISA, and surface staining of live *E. coli* O25b cells by flow cytometry. Sequencing of murine MAbs was performed by cloning cDNA specific to the VL and VH regions into commercial cloning vectors. Selected hybridoma clones were expressed as chimeric MAbs (i.e., the mouse variable regions were fused to human IgG1 constant domains and to kappa light chains). Three selected chimeric MAbs were subjected to humanization by CDR grafting technology (Fusion Antibodies Ltd., Belfast, United Kingdom). Briefly, the hypervariable (CDR) mouse antibody sequences were inserted into human framework sequences that were predicted *in silico* to be the most closely related to the original mouse frameworks. The best humanized MAb from each lineage was selected for further studies based on antigen binding affinity, surface staining, bactericidal activity, and *in vivo* protective efficacy. Antibodies were routinely expressed by CHO cells (Evitria AG, Schlieren, Switzerland) and purified through MabSelect or MabSelect SuRe resins. Aglycosylated MAb variants were generated by introducing N297Q mutations in the heavy chain.

Immunoblots. Immunoblotting was performed as described previously (30). Purified and separated LPS blotted onto polyvinylidene difluoride (PVDF) was reacted with 1 μg/ml of O25b-specific monoclonal antibodies or with LPS core-specific murine MAb WN1 222-5 (Hycult Biotech). Binding of MAbs was detected by horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ anti-human IgG (Southern Biotech) or goat $F(ab')_2$ anti-mouse IgG (Southern Biotech) at 1:40,000 dilution.

Biolayer interferometry (BLI). Antibody binding was measured by immobilizing biotinylated O25b polysaccharide antigen prepared as described previously (30) on streptavidin sensors (ForteBio, Pall Life Sciences) and monitoring the association of the MAbs (10 μg/ml) to the preloaded sensors for 10 min in DPBS containing 1% bovine serum albumin (BSA), followed by dissociation in the same buffer. The K_d (dissociation constant), k_{on} (association rate), and k_{off} (dissociation rate) values were determined using Data Analysis 7 software (ForteBio, Pall Life Sciences). Polyreactivity against nonrelated antigens was measured by immobilizing the MAbs on anti-human capture sensors (ForteBio, Pall Life Sciences) and monitoring the response for the association of 30 μg/ml antigen in solution for 10 min in PBS containing 1% BSA. Response values of <0.05 nm were considered negative. All polyreactivity antigens were purchased from Sigma-Aldrich.

Flow cytometry. Surface staining was performed as previously described (30). Overnight cultures of bacteria were diluted 1:100 in LB broth or in a 50% depleted heat-inactivated human serum pool and grown at 37°C to mid-log phase. Bacteria (10^6 CFU) were reacted with MAbs in the concentration range of 0.01 to 160 μg/ml, followed by staining with 4 μg/ml of Alexa Fluor 488-conjugated goat anti-human IgG secondary antibody (Life Technologies) and 5 μM SYTO-62 nucleic acid stain (Life Technologies). Samples were quantified in a BD Accuri C6 flow cytometer (BD Biosciences), and data were analyzed using FCS Express software version 4 (De Novo Software).

Serum bactericidal assay. Serum bactericidal assay (SBA) was performed in a 50% depleted human serum pool diluted with DPBS supplemented with calcium and magnesium. The reaction mixture contained ~5×10^3 CFU from LB broth- or serum-grown mid-log-phase bacterial suspension and 10 or 20 μg/ml MAb, respectively. Mixtures without any antibody and with isotype-matched irrelevant MAb were included as controls. Bacteria were enumerated by plating appropriate dilutions following 3 h (LB broth-grown bacteria) or 5 h (serum-grown bacteria) incubation at 37°C with shaking at 410 rpm. Killing mediated by specific MAbs was expressed as killing (%) = $100 - [(CFU_{MAb}/CFU_{control\ antibody}) \times 100]$.

Animal experiments. All animal experiments were performed according to Austrian law (BGBl. I Nr. 114/2012, approved by MA58, Vienna). Female 6- to 8-week-old BALB/cJRj mice (Janvier) were used in all experiments.

The protective efficacy of MAbs was assessed by intraperitoneal injection of MAbs diluted in DPBS in a total volume of 500 μl 24 h prior to challenge with a >90% lethal dose (determined in pilot studies) of *E. coli* 81009 or 3O strains. Control groups received isotype-matched (human IgG1) irrelevant MAb at the same dose. Challenge was performed intravenously with 100 μl of bacterial suspension. Bacteria were grown to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.5) in LB broth, washed with DPBS, and diluted to the target inoculum (1×10^9 CFU/ml for 81009 and 1.5×10^9 CFU/ml for 3O). Alternatively, bacteria were grown in 50% depleted and heat-inactivated human serum until mid-log phase (OD_{600} of ~0.2). Mice were challenged with serum-grown bacteria from frozen glycerol stocks washed and diluted in DPBS to 3×10^9 CFU/ml. In all cases, survival was monitored daily for 2 weeks. Statistical analysis was performed by the log rank (Mantel-Cox) test using GraphPad Prism 5.04 software. Differences were considered statistically significant when the P value was <0.05.

RESULTS

Binding characteristics of three humanized O25b-specific MAbs. First, O25b-reactive MAbs were generated with standard hybridoma technology by immunizing mice with *E. coli* ST131-O25b:H4 cells. Mouse MAbs displaying the best binding to purified LPS and live bacterial cells were selected for the generation of murine-human IgG1 chimeric antibodies, three of which were humanized by CDR grafting technology, as described in Materials and Methods.

The antigen specificities of humanized MAbs 3E9-11, 2A7-01, and 4D5-02 were demonstrated by immunoblotting using purified LPS. All selected MAbs bound to the O25b antigen, whereas no binding was observed to unrelated, i.e., non-O25 LPS, molecules (Fig. 1). MAb 4D5-02 cross-reacted with the O25a antigen, while the other two MAbs did not, suggesting that MAb 4D5-02 recognizes a different epitope that is shared by O25a and O25b.

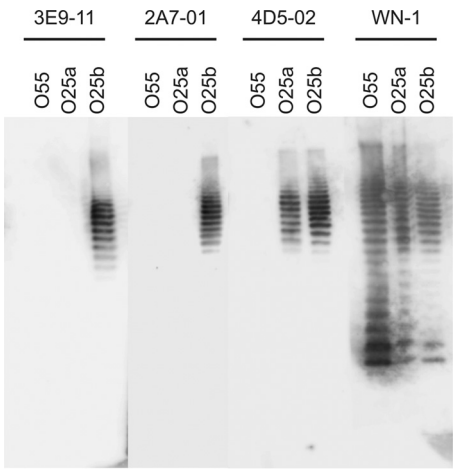

FIG 1 Immunoreactivity of O25b-specific humanized MAbs with purified LPS. Purified LPS samples from *E. coli* serotypes O25b, O25a, and O55 (control) were separated and blotted onto PVDF membranes that were reacted with the indicated MAbs. The inner-core-specific cross-reactive WN-1 222-5 MAb reacting with all LPS types was used as a positive control.

The binding characteristics were further investigated by biolayer interferometry (BLI; ForteBio) using biotinylated O25b polysaccharides. The affinity of all of the MAbs was in the range of 10^{-7} to 10^{-8} M (Table 1), which is in good agreement with values published for other anti-carbohydrate MAbs (31, 32). Importantly, no polyreactive characteristic for any of the MAbs was detected based on lack of binding to unrelated antigens, such as DNA, gelatin, fetuin, and dextran (Table 1).

Binding to the native O25b antigens on the surface of live *E. coli* cells was assessed by flow cytometry. We observed comparably high levels of surface binding to two different ST131-O25b:H4 strains expressing different capsule types with all three antibodies at concentrations of ≥20 μg/ml (Fig. 2A). At lower antibody concentrations, the 3E9-11 and 2A7-01 MAbs displayed significantly higher surface binding intensity than that found for 4D5-02. In addition, surface binding was confirmed using a panel of clinical ST131 isolates representing different pulsotypes (28). All pulsotypes, with the exception of pulsotype O (previously shown to express the O16 antigen), were strongly stained by all three MAbs (see Table S1 in the supplemental material).

Encouraged by the observation that Gram-negative bacteria grown under *in vivo*-like conditions demonstrate changes in gene expression (33, 34), particularly those involved in the synthesis and export of surface molecules, we also measured antibody binding to live *E. coli* cells grown in the presence of human serum. We observed reduced surface staining of serum-grown bacteria; however, it was still considered intense based on the 10- to 100-fold increase in median fluorescent intensity relative to the negative-control antibody (Fig. 2B).

***In vitro* bactericidal activity of O25b MAbs.** To measure the bactericidal activity attributable to the three humanized MAbs, an SBA was performed to detect antibody-mediated complement-dependent killing of *E. coli* cells. We observed an antibody concentration-dependent bacterial killing with all three antibodies (Fig. 3A), which was completely abolished upon heat inactivation of human serum (see Fig. S1 in the supplemental material). Consistent with the surface staining data, 3E9-11 proved to be the most efficacious MAb at low antibody concentrations, resulting in >95% killing compared to that of control antibody during a 3-hour incubation. The other two MAbs required higher concentrations (>10 to 20 μg/ml) to reach the same level of bactericidal efficacy.

Similarly, a highly efficacious bactericidal effect was observed when the assay was performed using bacteria grown in depleted human serum (Fig. 3B). Serum-grown bacteria required a higher antibody concentration and longer incubation (5 h versus 3 h) than LB broth-grown bacteria to give a comparable effect.

***In vivo* protection.** The protective efficacy of the selected humanized MAbs was tested in a murine bacteremia model using two unrelated ST131-O25b:H4 challenge strains. At an antibody dose of 100 μg/mouse (corresponding to ~5 mg/kg dose), all three tested MAbs provided comparable high levels of protection against both strains (Fig. 4A and B). Considering the differences between the three MAbs observed *in vitro*, we were interested in comparing the protective efficacies as a function of antibody concentration. Passive immunization of mice with MAb doses between 1 and 150 μg (corresponding to ~0.05 to 7.5 mg/kg and an estimated 1.3 to 200 μg/ml serum levels) revealed that MAb 3E9-11 was the most efficacious, while 4D5-02 was the least potent at lower antibody doses (Fig. 4B). This efficacy ranking was consistent with the *in vitro* bactericidal activities measured with the three MAbs (Fig. 3A).

In light of the lower surface-staining intensity and delayed bactericidal effect observed with serum-grown compared to LB broth-grown bacteria (Fig. 2B and 3B), we measured the protective efficacy of MAbs against bacteria preconditioned in human serum. Survival data from these experiments confirmed protection comparable to that observed for LB broth-grown bacteria at the same MAb dose (Fig. 4B and C).

In order to corroborate that the protection seen in the mouse

TABLE 1 Binding of O25b MAbs to the cognate antigen (O25b polysaccharide), expressed as affinity, association, and dissociation constants, and binding to unrelated antigens, expressed as response values

MAb	K_d (M^{-1})	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	Polyreactivity response (nm)			
				Dextran	DNA	Fetuin	Gelatin
3E9 chimeric	5.56E–08	2.83E+05	1.57E–02	−0.0114	−0.0114	−0.0076	−0.0159
3E9-11	6.94E–08	2.44E+05	1.69E–02	−0.0124	−0.0183	−0.0145	−0.016
2A7 chimeric	1.12E–07	1.42E+05	1.59E–02	−0.0178	−0.0175	−0.0146	−0.0161
2A7-01	1.15E–07	1.62E+05	1.86E–02	−0.0151	−0.0157	−0.0163	−0.0165
4D5 chimeric	1.49E–08	4.36E+04	6.51E–04	−0.0107	−0.0033	−0.0034	0.0024
4D5-02	3.82E–08	3.86E+04	1.47E–03	−0.0182	−0.0143	−0.0144	−0.0167
Irrelevant control MAb				0.0056	0.0121	−0.0224	−0.0164

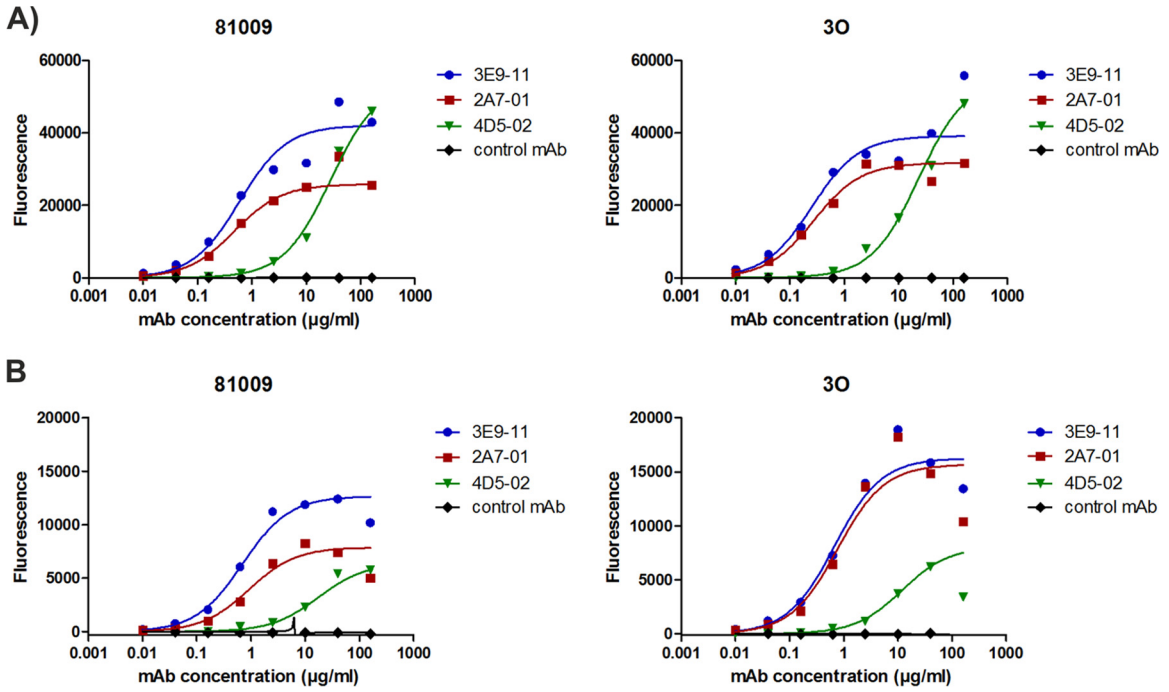

FIG 2 Surface staining of live *E. coli* cells with O25b-specific MAbs. Two *E. coli* ST131-O25b:H4 clinical isolates, 81009 and 3O, were grown to mid-log phase in LB broth (A) or in 50% depleted heat-inactivated human serum (B) and stained with MAbs at a concentration range of 0.01 to 160 μg/ml. Fluorescent intensity of bacteria was determined by flow cytometry using labeled secondary IgG.

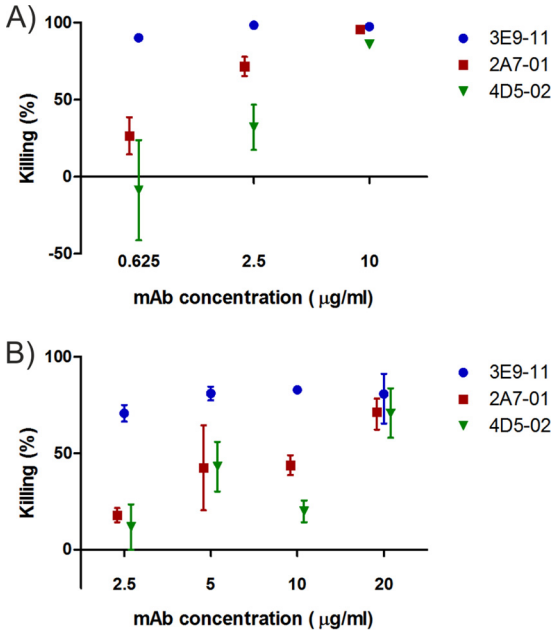

FIG 3 Serum bactericidal activity of O25b MAbs. Bactericidal effect was expressed as percent reduction of CFU relative to the bacterial numbers recovered from the control (irrelevant MAb) group. (A) Bacteria grown in LB broth incubated with humanized MAbs for 3 h. Combined results of 3 independent experiments shown as means ± standard errors. (B) Bacteria grown in 50% depleted heat-inactivated human serum incubated with humanized MAbs for 5 h. Combined results of 2 independent experiments shown as ranges (B).

model was mediated by the activation of complement, aglycosylated versions of the three MAbs were tested. These aglycosylated antibodies were generated by replacing the asparagine residue in the Fc domain of IgGs (N297Q) known to be essential for N-linked glycosylation and effective activation of the complement system via C1q binding. In these comparative studies, we used the minimal protective dose of each antibody, which was 5, 10, and 25 μg/mouse for 3E9-11, 2A7-01, and 4D5-02, respectively (based on data shown in Fig. 4B), or a 50-μg/mouse dose for all MAbs, which was considered to result in antibody excess. At the minimal protective MAb doses, the significant protection elicited by the glycosylated IgGs was lost upon use of the aglycosylated versions (Fig. 5A). Surprisingly, however, the protective capacity was found to be independent of the glycosylation status at the higher MAb dose (Fig. 5B).

To investigate this unexpected finding, we measured the *in vitro* bactericidal effect of aglycosylated MAbs side by side with the glycosylated (wild-type) counterparts. Surprisingly, at high doses, aglycosylated MAbs induced 70% to 92% of the killing, elicited by the glycosylated MAbs. Nevertheless, their bactericidal effect decreased rapidly upon dilution of the antibodies (Fig. 6).

DISCUSSION

Passive immunization with hyperimmune sera (serum therapy) was a standard treatment option in the preantibiotic era. As we move toward a possible post-antibiotic era, it may be prudent to reconsider the merits of this general approach. Current state-of-the-art biomedical research and biopharmaceutical manufacturing capabilities make it possible to generate highly purified human/humanized MAbs against a range of pathogenic microorganisms. MAbs directed against nonhuman targets and as a class of therapeutics in general have an excellent safety record and are well tolerated in the clinic (unlike therapy with serum). To

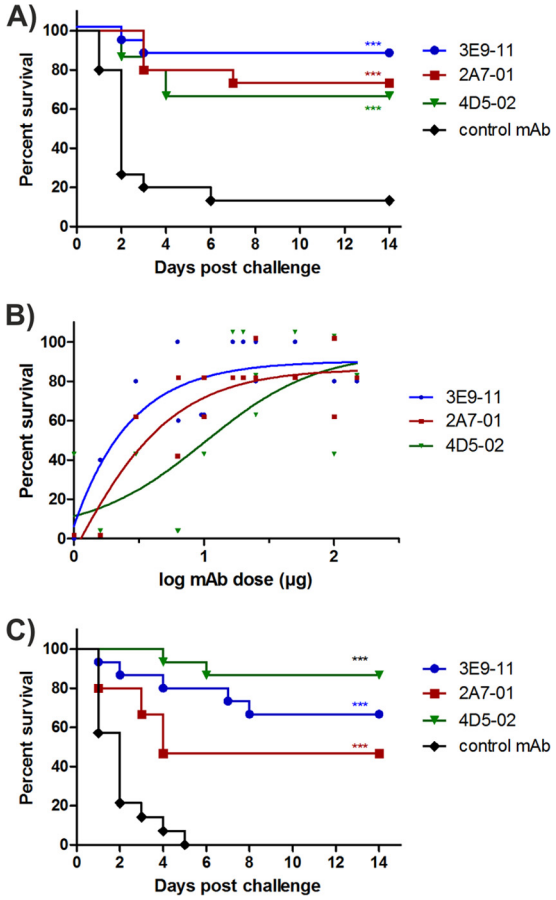

FIG 4 Protective efficacy of selected humanized MAbs in a murine lethal bacteremia model. Passive immunization was performed by intraperitoneal injections of MAbs 24 h prior to lethal intravenous challenge with strain 3O grown in LB broth (A), strain 81009 grown in LB broth (B), or strain 81009 grown in depleted human serum (C). Doses of 100 μg/mouse were used routinely, and survival curves for 14 days are depicted in panels A and C. Dose response curves with three selected MAbs within the range of 1 to 150 μg were performed, and survival rates over control at day 14 postinjection are plotted in panel B. Combined results of 2 (A) or 3 (C) independent experiments are shown. *P* values were calculated using the log rank test (***, $P < 0.001$).

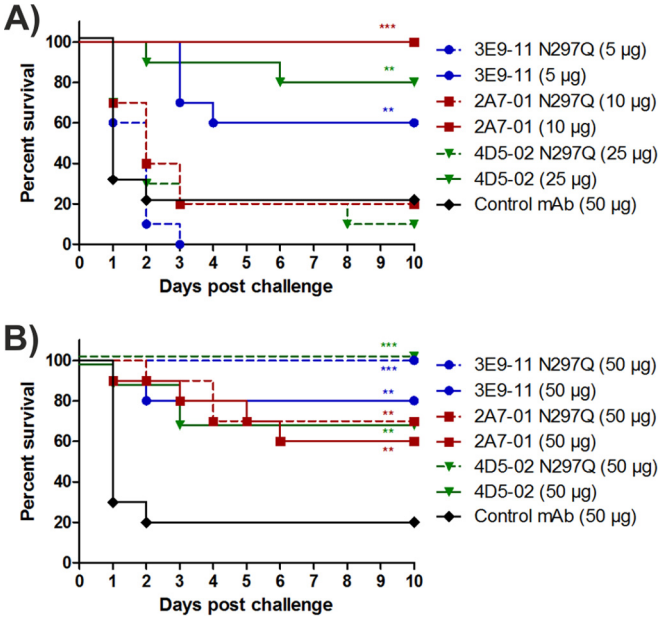

FIG 5 *In vivo* efficacy of aglycosylated MAbs. Mice were passively immunized with a minimal protective dose (A) or an excessive dose (B) of three different O25b-specific MAbs and their aglycosylated variants. Survival curves following a subsequent lethal challenge by strain 81009 are shown. Graphs show combined results of 2 independent experiments with groups of 5 mice each. *P* values (log rank test) are shown where survival was significantly different relative to the control group (**, $P < 0.01$; ***, $P < 0.001$).

date, there are two licensed MAb products against infectious targets (35, 36) and many more in the clinical phase of testing or under preclinical development (37, 38).

The challenges associated with the development of antibacterial antibodies include finding molecular targets that are accessible on the surface of a given bacterium and simultaneously shared by clinically relevant strains. Since conserved bacterial surface molecules are masked by highly variable surface polysaccharides in *E. coli* (and other enterobacterial pathogens), it is difficult to find a target that can provide broad-spectrum protection. The concept of "magic bullets" that are able to treat a broad variety of pathogens might be considered unrealistic and should be replaced by rational designs of species/subspecies-specific therapeutic approaches. These novel antimicrobials with targeted coverage are not expected to induce widespread resistance or to have detrimental effects on normal microbiota.

In this paper, we described highly efficacious MAbs targeting the unique O antigen (30) of a clinically relevant MDR *E. coli* clonal lineage. The humanized MAbs binding to the O25b antigen were shown to confer high levels of protection in a murine model of bacteremia. This is in accordance with previous reports suggesting that the LPS O antigen is a protective antigen, i.e., that antibodies against these epitopes can elicit protection (23, 24, 39, 40).

The primary aim of the study was to identify MAbs capable of triggering bactericidal effects mediated solely by the complement system, i.e., without the involvement of phagocytes. Activation of the classic complement pathway is initiated by the binding of C1q to the antigen-bound antibody complex. C1q is a hexamer molecule that requires binding to several Fc regions for efficient activation of the complement cascade. This is supported by the greater complement-activating potential of pentameric IgM molecules compared to that of IgG subclasses. In order to be able to develop therapeutic MAbs of the IgG isotype with such an intended mode of action, we considered it important to select a target antigen that is highly abundant on the surface. We envisioned that adjacent binding of multiple IgG molecules may efficiently trigger the binding of C1q and hence activate the classic complement pathway. Indeed, the main mode of action of O25b-specific MAbs appears to be complement-mediated killing, as confirmed both *in vitro* and *in vivo*. *In vivo*, at minimal protective doses, aglycosylation of MAbs eliminated protection, confirming an Fc-mediated mode of action. Interestingly, however, at excessive MAb doses, even aglycosylated MAbs provided protection. One possible explanation for this protection is that aglycosylated MAbs retain some residual capacity of complement activation. This is supported by previous reports showing ~30% residual binding of C1q and the consequent ability of aglycosylated MAbs to lyse sensitized erythrocytes at higher concentrations (41, 42). Our data

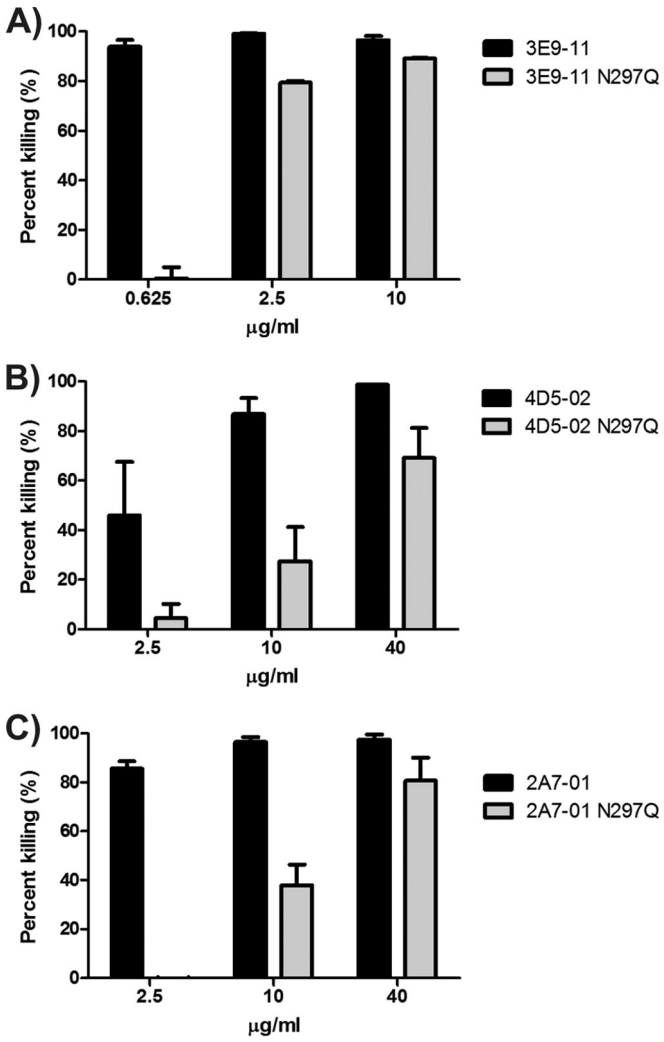

FIG 6 *In vitro* bactericidal efficacy of aglycosylated MAbs. Bactericidal activities of three aglycosylated MAbs were determined simultaneously with their glycosylated counterparts. Bactericidal activity is expressed as percent killing, i.e., CFU recovered in test groups relative to the control group (irrelevant MAb control) following 3 h of incubation in 50% depleted human serum. (A) MAb 3E9-11; (B) MAb 4D5-02; (C) MAb 2A7-01. Black bars, wild-type MAbs; gray bars, Fc mutated to remove glycosylation. Means with range for 2 independent assays are shown.

showed an ~10-fold reduction in bactericidal effect *in vitro* and a 2- to 10-fold higher MAb dose required for *in vivo* protection with aglycosylated MAbs, which showed good correlation with the previous results. Nevertheless, other studies showed a complete loss of C1q binding for aglycosylated MAbs (43). We hypothesize that the level of abundance of the different target antigens may at least partially explain these seemingly contradictory results. Nevertheless, we cannot rule out that the efficacy of a large amount of aglycosylated MAbs originates from involvement of other complement pathways or via other modes of action *in vivo* (e.g., agglutination or endotoxin neutralization). Further animal experiments (in murine and nonmurine models) and *in vitro* assays are needed to clarify these possibilities. Still, the principal mode of action for protection by the O25b-specific MAbs described in this study seemed to be the antibody-dependent complement-mediated bactericidal effect that was confirmed by *in vitro* experiments.

We considered it important to test O25b-specific MAbs against bacteria grown under *in vivo*-like conditions. Investigating bacterial pathogens cultured in common laboratory media may result in observations that are not directly relevant to the clinic because of the differences in the types of bacterial antigens expressed *in situ* under different environmental conditions. Recently, a transcriptome analysis of *E. coli* grown in serum versus that grown in LB broth revealed that the envelope substantially realigned when cultured in serum (33, 34). Importantly, while the accessibility of O25b antigens was lower when grown in serum (as shown by flow cytometry) due to the abundance of LPS, avid binding of MAbs to this target may still mediate a bactericidal effect. In the mouse protection studies, as little as 100 μg of the MAbs was shown to be highly protective against a challenge with 3×10^8 CFU of serum-grown mid-log-phase *E. coli* cells (and even lower doses against LB broth-grown bacteria). This MAb dose corresponded to 5 mg/kg, a relatively low concentration compared to other anti-infective MAbs tested in preclinical experiments (37, 38) or clinical trials (35, 36). Furthermore, in human bacteremia, the live bacterial numbers rarely exceeded 10^3 to 10^4 CFU/ml of blood (44).

Since there are ~180 structurally and hence antigenically different O types of *E. coli*, the O25b-specific MAbs target only those *E. coli* cells that express this particular LPS antigen. However, the overrepresentation of isolates belonging to the ST131-O25b:H4 clonal group among MDR extraintestinal infections still justifies development of a MAb directed against this target. It was demonstrated that within the ST131 lineage, all strains that expressed the O25b antigen, irrespective of capsular and pulsotype, were bound by the tested MAbs.

Given the targeted specificity, a companion diagnostic tool that can rapidly identify infections by representatives of this clone would present a significant clinical advantage and cost benefit. Recently, we published a prototype of a bead-based agglutination assay that, with further development, may be appropriate for this purpose (30). Since this highly specific and sensitive diagnostic tool also utilizes O25b-specific MAbs, it would identify only isolates that in fact express the O25b antigen (in contrast to genotyping) and so would reliably identify patients who might benefit from passive immunotherapy with O25b-specific MAbs.

We envision that MAbs may provide a therapeutic alternative for strains resistant to available antibiotics. Although carbapenem-resistant *E. coli* strains are currently not prevalent, this may rapidly change, as exemplified by the related pathogen *Klebsiella pneumoniae*. On the other hand, LPS-specific MAbs may exert endotoxin-neutralizing potential and hence complement antibiotic therapy. Such a synergistic mode of action may be desirable given the concerns with LPS release associated with some antibiotics (45, 46). Experiments aiming to show additive and/or synergistic effects of antibiotics and MAbs are ongoing.

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