Lysibodies are IgG Fc fusions with lysin binding domains targeting *Staphylococcus aureus* wall carbohydrates for effective phagocytosis

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The cell wall of Gram-positive bacteria contains abundant surface-exposed carbohydrate molecules that are highly conserved within and often across species. The potential therapeutic usefulness of high-affinity antibodies to cell wall carbohydrates is unquestioned, however obtaining such antibodies is challenging due to the poor overall immunogenicity of these bacterial targets. Autolysins and phage lysins are peptidoglycan hydrolases, enzymes that have evolved over a billion years to degrade bacterial cell wall. Such wall hydrolases are modular enzymes, composed of discrete domains for high-affinity binding to cell wall carbohydrates and cleavage activity. In this study, we demonstrate that binding domains from autolysins and lysins can be fused to the Fc region of human IgG, creating a fully functional homodimer (or "lysibody") with high-affinity binding and specificity for carbohydrate determinants on the bacterial surface. Furthermore, we demonstrate that this process is reproducible with three different binding domains specific to methicillin-resistant *Staphylococcus aureus* (MRSA). Cell-bound lysibodies induced the fixation of complement on the bacterial surface, promoted phagocytosis by macrophages and neutrophils, and protected mice from MRSA infection in two model systems. The lysibody approach could be used to target a range of difficult-to-treat pathogenic bacteria, given that cell wall hydrolases are ubiquitous in nature.

immunotherapy | antibody | vaccine | monoclonal | peptidoglycan hydrolase

The rise of multidrug-resistant bacteria has created a need for alternatives to conventional antibiotics. One important approach is the use of therapeutic antibodies, which have recently become a mainstay in areas such as cancer therapy and inflammation and are now increasingly being developed to treat infectious diseases (1–3). Most of the latter antibodies developed thus far target virulence factors that are either secreted or bound to the bacterial surface; however, creation of opsonic antibodies to the carbohydrate components exposed on the bacterial surface remains an important yet elusive goal of immunotherapy. Carbohydrates are a major component of the Gram-positive bacterial cell wall (up to 60% in dry weight) and are invariant within and often across bacterial species. Cell wall carbohydrates are often surface-exposed and required for proper cell wall function (4–8). Although carbohydrates are attractive targets for the development of therapeutic antibodies, their structures are poor immunogens because they are T cell-independent antigens. Thus, they elicit an immune response characterized by the production of low-affinity IgMs, the absence of class-switched antibodies and memory, and a short half-life (9–11). One approach that can promote effective immunity to carbohydrates is the creation of protein–carbohydrate conjugates; recent advances in glycobiology and chemical synthesis of carbohydrates have increased the scope of this method (11). This approach, though complex and expensive, has enabled the development of effective vaccines against certain carbohydrates such as capsular polysaccharides, however capsules are often variable and require the production of a polyvalent vaccine for effective protection (12). As such, proteins represent the major class of molecular targets for antibody therapies, and attempts to target carbohydrates have been less successful (11).

Although high-affinity antibodies to cell wall carbohydrates are rare, cell wall hydrolases, which are ubiquitous in nature, can bind with very high affinity (13–15). For example, bacteria produce cell wall hydrolases (autolysins) to separate daughter cells following division and facilitate peptidoglycan turnover (16), and bacteriophages produce wall hydrolases (lysins) to release progeny from infected bacterial hosts (14, 15). Both autolysins and lysins have discrete cell wall carbohydrate binding domains required for proper function. By creating IgG Fc fusions with binding domains from different cell wall hydrolases, we produced "lysibodies" with specificity toward bacterial cell wall carbohydrate epitopes.

As a proof-of-concept, we produced lysibodies specific for the cell wall of *Staphylococcus aureus*. *S. aureus* is a leading cause of skin and soft tissue infection as well as a diverse array of severe invasive infections, making it one of the major causes of pathogen-related death in the United States (17–20). The rise in antibiotic resistance is a major concern that is not adequately addressed by the anti-infective development pipeline. In particular, methicillin-resistant *S. aureus* (MRSA) is now prevalent in both the hospital and community settings, representing an enormous public health burden worldwide (17, 21–23). Vaccines and therapeutic antibodies represent a prominent alternative to antibiotics; however, to date,

Significance

Antibiotic resistance is an ever-increasing problem; for certain pathogens, few treatment options remain. Vaccines and therapeutic antibodies represent important alternatives to antibiotics, yet despite extensive effort no approved vaccine is available for *Staphylococcus aureus*. Although wall carbohydrates are ideal immunotherapeutic targets due to their abundance and high level of conservation, their poor immunogenicity compared with conventional protein targets complicates the production of effective antibodies. The approach presented here fuses the high-affinity binding domains from bacteriophage lysins and autolysins that recognize specific cell wall carbohydrate epitopes to IgG Fc, creating effective therapeutic antibodies, or lysibodies. This approach is generalizable, allowing production of antibodies to poorly immunogenic carbohydrate epitopes of many Gram-positive pathogens. Lysibodies thus represent a broad class of anti-infectives.

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none has successfully reached regulatory approval for *S. aureus* (24–27). By combining the *S. aureus*-specific carbohydrate binding activity of selected autolysins and lysins with the Fc effector functions of human antibodies, we created three lysibodies specific to *S. aureus*. These lysibodies recognized a range of clinically important staphylococcal strains, fixed complement on the bacterial surface, induced phagocytosis by macrophages and neutrophils, and protected mice from challenge with MRSA in two model systems.

Results

Lysibody Construction and Production. IgG antibodies are composed of two heavy chains and two light chains, stabilized by disulfide bridges and noncovalent interactions. Each antibody can be functionally divided into two Fab fragments, which bind to target epitopes, a hinge region, and an Fc fragment. The Fc fragment binds to a diversity of Fc receptors, including FcRn and type I and II FcγRs, and thus determines half-life and mediates effector functions that lead to the elimination of pathogens (28). Lysibodies were produced by fusing a human IgG1 Fc with a cell wall binding domain of staphylococcal autolysin or phage lysin. Lysibodies contain a leader sequence to promote secretion and a hexahistidine tag for purification. Cysteine 220 of human IgG1 heavy chain, which in the native molecule forms a disulfide bridge with the light chain, was mutated to glycine, as a light chain is not present in lysibodies; cysteines required for the formation of disulfide bridges between the two heavy chains were retained. Thus, the final lysibody structure is a two-chain homodimer (Fig. 1*A*).

The AtlA lysibody was created by replacing the V_H and C_{H1} domains of human IgG1 heavy chain with the R1–R2 binding repeats of the major staphylococcal autolysin AtlA (16, 29). AtlA binds *S. aureus* lipoteichoic acid (LTA) on the cell surface (30), which is essential for *S. aureus* survival (31). AtlA binding repeats R1–R2 bind with high affinity at $\sim 10^8$ binding sites per staphylococcal cell, predominantly in the vicinity of the division ring (29, 32). As control, we produced a construct with a single chain fragment variable (Fv) specific for chicken ubiquitin in place of the AtlA binding repeats, termed "ChUb construct" (Fig. 1*B*).

A slightly different approach was used to produce lysibodies containing binding domains from phage lysins. Unlike autolysins, the cell wall binding domain of most phage lysins is found at the C terminus of the molecule (33). Placing such domains at the N terminus of an antibody Fc region (directly replacing the Fab) results in an unnatural orientation for the lysin binding domain, potentially interfering with its function. As there are numerous examples of functional antibodies with C-terminal fusions (34), we created lysibodies using the binding domains of the *S. aureus*-specific lysins ClyS (35) and PlySs2 (36) fused to the C terminus of human IgG1 Fc region (Fig. 1*B*). As controls we also produced a lysibody containing the binding domain of the *Bacillus anthracis*-specific lysin PlyG (37) and a construct containing no binding domain whatsoever ("Fc only"; Fig. 1*B*). We used I-TASSER (38) to perform structural predictions on lysibody monomers. Each predication showed an extended structure with clear domain delineation for lysibodies, resembling the control ChUb construct—that is, a typical antibody with a single chain Fv (Fig. S1). Formation of Fc dimers would likely further stabilize these predicted structures in the correct domain organization.

Lysibodies were produced in mammalian cells to allow glycosylation of the Fc fragment, required for effector functions. Like typical single-chain antibodies, lysibodies formed the expected dimers that were stabilized by disulfide bridges, as determined by SDS/PAGE and Western blot analysis (Fig. 1*C*). Elimination of disulfide bridges with β-mercaptoethanol (BME) resulted in products with one-half the molecular mass. The binding avidity of lysibodies is predicted to be significantly higher than that of the original phage lysin or autolysin as a result of dimerization.

We used fluorescence microscopy to test the binding of lysibodies to the cell wall of *S. aureus*. Initially, we used the protein A negative *S. aureus* strain Wood 46 to avoid nonspecific binding of protein A to the Fc region of lysibodies. Although protein A needs to be addressed when studying lysibody binding in vitro using purified reagents, it is less likely to inhibit lysibody activity in the blood environment (39); human serum contains abundant nonspecific antibodies that saturate protein A, as shown in Fig. S2. AtlA lysibody showed extensive labeling of the staphylococcal cell wall with some preference for the septa (Fig. 1*D*), similar to previous observations (29, 32). No signal was detected with the ChUb construct or the PBS control. Similarly, C-terminal fusion ClyS and PlySs2 lysibodies bound *S. aureus*, whereas the Fc-only construct showed no binding (Fig. 1*E*). The anthrax-specific PlyG lysibody showed a very slight cross-reactivity with *S. aureus*.

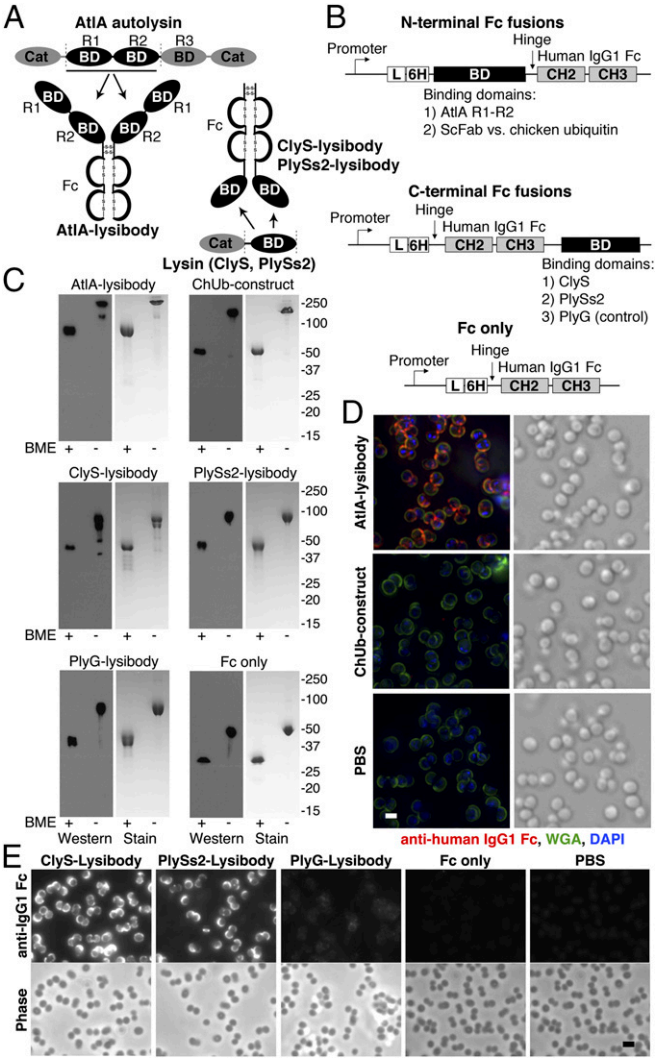

Fig. 1. Lysibody construction, dimerization, formation of disulfide bridges, and binding to target organisms. (*A*) Schematic representations of lysibody structure. BD, binding domain; Cat, catalytic domain. (*B*) Structure of the expression vectors for lysibodies and controls. L, leader sequence; 6H, hexahistidine tag. (*C*) Purified lysibodies were run on 10% SDS/PAGE with or without the reducing agent BME and analyzed by Western blot using anti-human IgG1 Fc antibody. A duplicate gel was stained with Coomassie blue. (*D*) Binding of AtlA lysibody to *S. aureus* Wood 46 (protein A negative) was determined by deconvolution immunofluorescence microscopy. Maximum intensity projections are presented. Anti-human IgG Fc Alexa Fluor 594 conjugate, red; wheat germ agglutinin (WGA), green; DAPI, blue (scale bar, 1 μm). (*E*) Binding of C-terminal Fc fusion lysibodies to *S. aureus* Wood 46 was determined by immunofluorescence microscopy, using anti-human IgG Fc Alexa Fluor 594 conjugate (scale bar, 2 μm). Experiments were repeated three times. ScFab, single chain fragment antigen-binding.

Target Binding Range of Lysibodies. We evaluated the binding of lysibodies to various methicillin-resistant, vancomycin-intermediate, and vancomycin-resistant *S. aureus* strains (MRSA, VISA, and VRSA, respectively) using fluorescence microscopy. To avoid possible nonspecific interaction of the Fc portion of lysibodies with staphylococcal protein A, we first blocked protein A with goat and human serum and used AtlA lysibody or ChUb construct that was directly labeled with Rhodamine red. To determine the binding range of ClyS and PlySs2, we used fusion proteins of the lysin binding domain and green fluorescent protein (GFP). The binding domains of AtlA, ClyS, and PlySs2 bound all *S. aureus* clinical isolates tested, although some variability in fluorescent signal was observed; controls did not bind any of the strains tested (Figs. S3 and S4).

We further characterized the binding range of lysibodies to various staphylococcal species and other bacteria. AtlA lysibody, ClyS lysibody, and PlySs2 lysibody bound *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hyicus*, and *Staphylococcus sciuri*, in addition to *S. aureus* (Figs. S5 and S6). For AtlA lysibody and ClyS lysibody, slight to no signal was observed for nonstaphylococcal species (AtlA lysibody bound weakly to *Bacillus cereus* and *Enterococcus faecalis*, exclusively at the septum). PlySs2 lysibody, on the other hand, displayed a broad range of host binding, including staphylococci, *E. faecalis*, *Enterococcus faecium*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*, consistent with the wider lytic activity range of the PlySs2 lysin (36). PlyG lysibody specifically bound *B. anthracis* (Fig. S6).

Lysibodies Induce Phagocytosis of *S. aureus* by Macrophages. Killing of *S. aureus* by phagocytes is an important immunological mechanism controlling staphylococcal infections. To determine the extent of lysibody-induced phagocytosis, cells of the GFP-expressing *S. aureus* strain Newman/pCN57 were incubated with a monolayer of macrophages for an hour in the presence of various lysibodies. Macrophages were washed and analyzed by microscopy (Fig. 2*A* and Fig. S7) and flow cytometry (Fig. 2 *B–D*) to determine the extent of phagocytosis. We first gated on the macrophage population and then determined the percentage of highly fluorescent macrophages, indicating a substantial staphylococcal load (Fig. 2*B*). AtlA lysibody induced phagocytosis of staphylococci in a dose-dependent manner by both Raw 264.7 cell line macrophages and primary peritoneal murine macrophages, whereas the ChUb construct and the nonspecific monoclonal antibody 1K8 had no effect (Fig. 2*C*). Similarly, ClyS and PlySs2 lysibodies induced phagocytosis of *S. aureus* in a dose-dependent manner, whereas the *B. anthracis*-specific PlyG lysibody had only a minimal effect at the highest concentration, possibly due to the low cross-reactivity with *S. aureus* carbohydrate (Fig. 1*E* and 2*D*). To determine whether staphylococci are internalized or merely attached to the surface of macrophages, we analyzed samples treated with ClyS lysibody or PlyG lysibody using deconvolution fluorescence microscopy, which allows 3D evaluation of the cells (Fig. S7). This analysis showed a much higher bacterial load in macrophages in the presence of ClyS lysibody (Fig. S7*A*). Examination of sequential Z sections allowed a clear distinction between intracellular and surface-attached staphylococci (Fig. S7*B*). Analysis of a large population of cells showed that in the presence of ClyS lysibody the staphylococcal load per macrophage increased dramatically compared with PlyG lysibody (Fig. S7*C*). Roughly 75% of the staphylococci observed were inside macrophages, whereas 25% were associated with the surface or partially internalized (Fig. S7*D*). We next determined whether lysibodies lead to the killing of phagocytosed staphylococci. Log phase *S. aureus* strain Newman cells were mixed with various lysibodies and incubated with Raw 264.7 macrophages in suspension for 3 h, at a 1:1 ratio of staphylococci to macrophages. Macrophages were then lysed and the number of bacterial colony-forming units (cfus) was determined through serial dilution and plating (Fig. 2*E*). Under the conditions tested, AtlA ClyS and

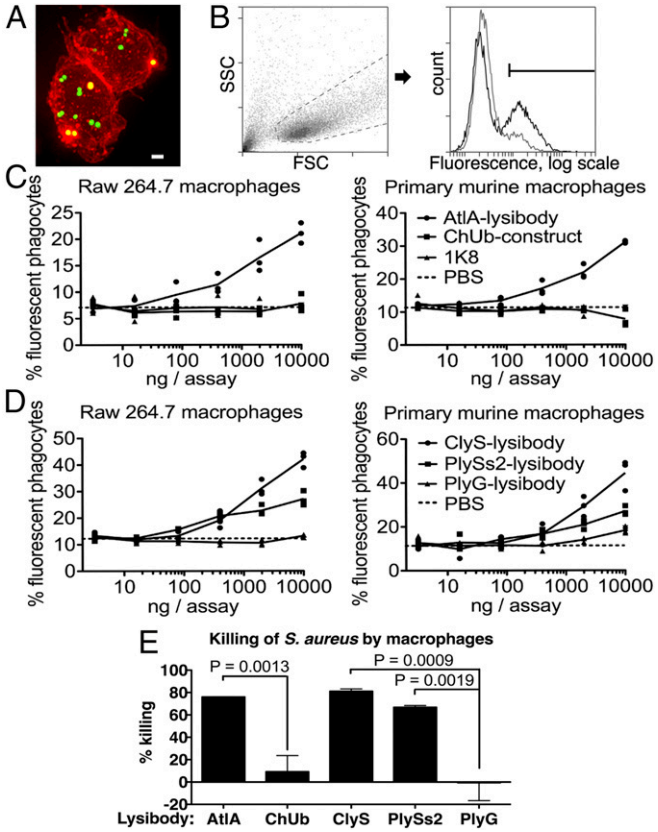

Fig. 2. AtlA lysibody induces phagocytosis of *S. aureus* by macrophages. Adherent macrophages were incubated for 1 h with fluorescent *S. aureus* Newman/pCN57 (GFP) in the presence of various lysibodies. The cells were washed, fixed, and analyzed by microscopy and flow cytometry. (*A*) A representative Raw 264.7 macrophage containing fluorescent staphylococci following ClyS lysibody treatment. Staphylococci, green; WGA Alexa Fluor 594 conjugate, red. The image is presented as a maximum intensity projection (scale bar, 2 μm). Also see Fig. S7. (*B*) Gating scheme for flow cytometry analysis: gating on macrophages using forward and side scatter (*Left*), followed by determination of the percentage of highly fluorescent macrophages (*Right*). Black, AtlA lysibody; gray, PBS. (*C*) Effect of lysibody dose on phagocytosis using the N-terminal fusions AtlA lysibody and ChUb construct and 1K8 nonspecific humanized monoclonal. (*D*) Effect of lysibody dose on phagocytosis using C-terminal fusions: ClyS lysibody, PlySs2 lysibody, and PlyG lysibody. Experiments were performed in triplicates and repeated three times. (*E*) Percent killing of *S. aureus* Newman by Raw 264.7 macrophages in the presence of 10 μg of various lysibodies, compared with PBS control. Experiments were performed in triplicates, with two technical repeats for each biological repeat; *P* values were calculated using *t* test. FSC, forward scatter; SSC, side scatter.

PlySs2 lysibodies led to killing of 65–80% of staphylococci compared with the PBS control. The relative killing efficiency ratios were ClyS lysibody > AtlA lysibody > PlySs2 lysibody, consistent with the results obtained in flow cytometry assays. The ChUb construct and PlyG lysibody controls did not lead to significant killing of staphylococci.

Lysibodies Fix Complement on the Surface of *S. aureus*. Complement deposition on the surface of pathogens is an important mechanism leading to their efficient removal by phagocytes. We used fluorescence microscopy to determine whether lysibodies can induce fixation of complement fragment C3b on the surface of *S. aureus*. Protein A negative strain Wood 46 was used to prevent a nonspecific fluorescent signal. Human serum that was preadsorbed on *S. aureus* to remove possible antibodies specific to this organism was used as a source of complement. AtlA, ClyS, and PlySs2 lysibodies induced complement deposition on the surface of the

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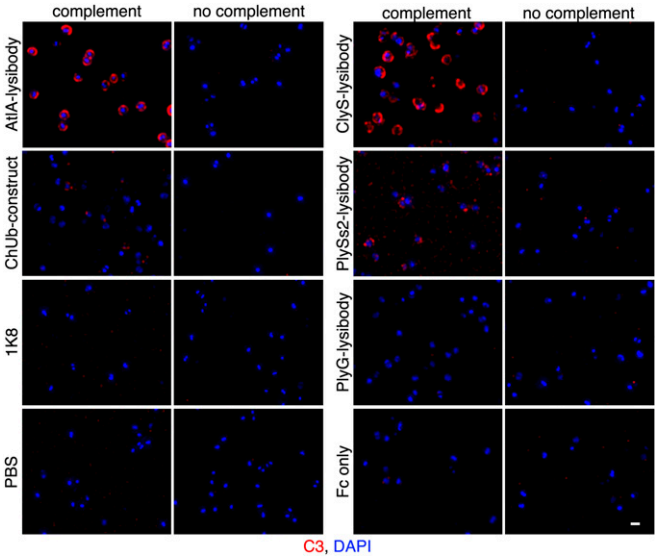

Fig. 3. Lysibodies induce deposition of complement on the surface of *S. aureus* cells. *S. aureus* Wood 46 cells (protein A negative) were attached to poly-L-lysine–coated coverslips and incubated with lysibodies. The cells were then incubated with human complement, washed, fixed, and blocked. Complement was detected using rabbit anti-C3, followed by Alexa Fluor 594 conjugate; DNA was stained with DAPI. Images were obtained using deconvolution microscopy and are presented as maximum intensity projections. Experiments were repeated twice (scale bar, 1 μm).

cells, although PlySs2 lysibody was less potent than the other two lysibodies (Fig. 3). Nonspecific controls (ChUb construct, 1K8 monoclonal antibody, PlyG lysibody, and Fc alone) did not induce complement deposition. For AtlA lysibody we also determined the extent of complement deposition on strain Newman/pCN57 using microscopy (Fig. S8*A*) and flow cytometry (Fig. S8*B*); protein A was blocked subsequent to complement deposition to prevent nonspecific fluorescent signal. This analysis demonstrated that induction of complement deposition is dose-dependent.

Lysibodies Induce *S. aureus* Phagocytosis by Neutrophils. Neutrophils are the first line of defense against *S. aureus* infection (40). Recognition of Fc by FcγRs and recognition of surface-attached C3b by C3 receptors are necessary for efficient phagocytosis by neutrophils. We evaluated the phagocytosis of fluorescent *S. aureus* by human neutrophils using fluorescence microscopy (Fig. 4*A* and Fig. S9) and flow cytometry (Fig. 4 *B–E* and Fig. S10). We used FITC-labeled staphylococci for increased sensitivity, as neutrophils typically phagocytosed fewer staphylococci per cell compared with macrophages. *S. aureus*-specific lysibodies (AtlA, ClyS, and PlySs2) induced phagocytosis of *S. aureus* USA300 by differentiated HL-60 neutrophils in a complement-dependent manner, whereas control constructs had no effect (Fig. 4*C*). Induction of phagocytosis was dose-dependent in all cases (Fig. 4*D*). AtlA lysibody and ClyS lysibody also induced phagocytosis of *S. aureus* in a complement-dependent manner using peripheral blood human polymorphonuclear cells, however PlySs2 lysibody was less effective with these cells (Fig. 4*E*). Similar results were obtained with *S. aureus* Wood 46 and USA600 (Fig. S10).

To rule out the possibility that the flow cytometry results represent bacteria attached to the surface of neutrophils rather than phagocytosed bacteria, we analyzed samples treated with AtlA lysibody or controls by both flow cytometry and deconvolution fluorescence microscopy for 3D evaluation of the cells (Fig. S9). The percentage of neutrophils containing intracellular bacteria using this method closely resembled the percentage obtained using flow cytometry. Furthermore, only a minority of staphylococci were observed attached to the surface of neutrophils, and these were often associated with phagocytic cups.

Lysibodies Protect Mice from *S. aureus* Infection. Two infection models were used to test protection of mice from *S. aureus* infection. In a kidney abscess model, 1 mg ClyS lysibody, the *B. anthracis*-specific PlyG lysibody, and PBS control were each injected intraperitoneally to BALB/c female mice. Twenty-four hours later, the mice were challenged intraperitoneally with a sublethal dose of 2.5×10^6 cfu of the methicillin-resistant, VISA strain USA600. Four days later, surviving mice were euthanized, and the bacterial load in the kidneys was determined through homogenization, serial dilution, and plating. Mice treated with ClyS lysibody had a significantly reduced bacterial load compared with mice treated with PlyG lysibody or PBS (Fig. 5*A*).

To test protection from bacteremia, 0.3 mg AtlA lysibody and PBS control were each injected intraperitoneally to female BALB/c

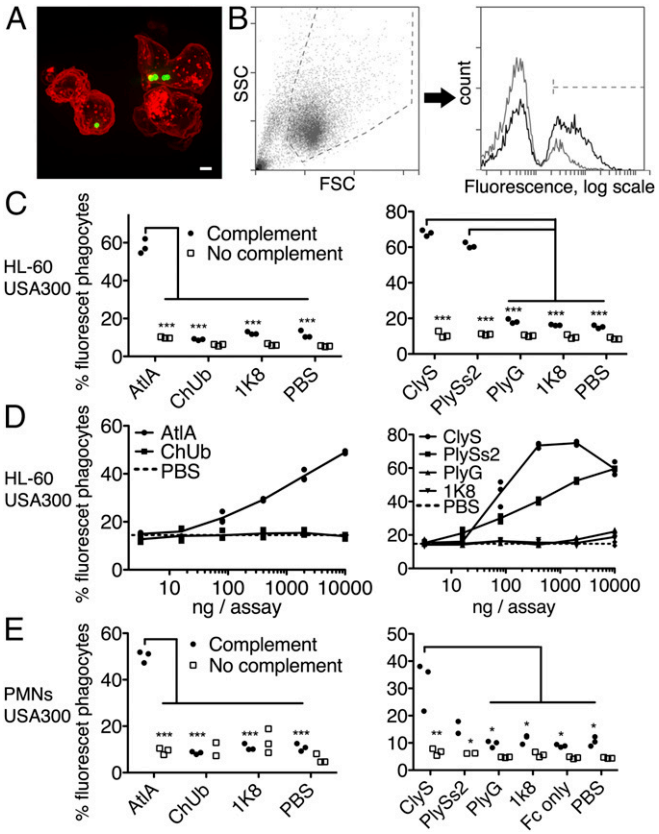

Fig. 4. Lysibodies induce phagocytosis of *S. aureus* by neutrophils. HL-60 neutrophils (*A–D*) and human polymorphonuclear cells (*E*) were incubated with FITC-labeled *S. aureus* strain USA300 in the presence of lysibodies and *S. aureus*-adsorbed human complement. (*A*) A representative image of HL-60 neutrophils incubated with FITC-labeled *S. aureus* USA300 and AtlA lysibody; a maximum intensity projection is presented (scale bar, 2 μm). Also see Fig. S9. (*B*) Gating scheme for flow cytometry analysis: gating on neutrophils using forward and side scatter (*Left*) and determination of the percentage of fluorescent neutrophils (*Right*). Black, AtlA lysibody; gray, PBS. (*C*) Phagocytosis of *S. aureus* by HL-60 neutrophils using 5 μg AtlA lysibody, ClyS lysibody, PlySs2 lysibody, or controls in the presence or absence of complement. (*D*) Effect of lysibody dose on *S. aureus* phagocytosis by HL-60 neutrophils. (*E*) Phagocytosis of *S. aureus* by human polymorphonuclear cells using 5 μg AtlA lysibody, ClyS lysibody, PlySs2 lysibody, or controls in the presence or absence of complement. All experiments were done in triplicates and repeated two to four times. Statistical significance analysis using the *t* test was performed for the relevant samples. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Also see Fig. S10 (Wood 46 and USA600). FSC, forward scatter; PMNs, polymorphonuclear leukocytes; SSC, side scatter.

mice. Twenty-four hours later, the mice were challenged intraperitoneally with 2×10^6 cfu of the MRSA strain MW2 (USA400). Mouse viability was monitored for 8 d, at which time surviving mice were euthanized. AtlA lysibody-treated mice had significantly improved survival rates compared with control mice (Fig. 5*B*).

Discussion

In this study, we report the development of a solution to a long-standing problem in immunology—how to create high-affinity opsonic antibodies to invariant and abundant bacterial cell wall carbohydrates. We demonstrate that binding domains from cell wall hydrolases can direct the Fc portion of an antibody to bacterial wall carbohydrates, enabling the fusion homodimer to function like a normal antibody: efficiently binding, opsonizing, inducing complement fixation, promoting phagocytosis of bacteria by macrophages and neutrophils, and protecting animals in infection models. As proof of concept, we produced three different lysibodies specific for *S. aureus*, using the binding domains from either the major staphylococcal autolysin AtlA, or two phage lysins—ClyS and PlySs2.

The major advantage of lysibodies compared with typical monoclonal antibodies is the ability to bind abundant carbohydrate targets on the bacterial wall that are highly conserved and thus unlikely to mutate to avoid binding. Many surface carbohydrates are critical for proper cell wall function (7). In *S. aureus*, the membrane-bound carbohydrate LTA is essential (31), and although mutants lacking wall teichoic acid (WTA) are viable, they have impaired pathogenicity and are less able to colonize the host (41). When using a binding domain derived from the pathogen's own autolysin, as in the case of the AtlA lysibody, mutations that would prevent lysibody binding would necessarily also disturb the action of the native autolysin, interfering with cell division. Resistance to lysibodies containing a binding domain from a phage lysin is also unlikely; lysins have evolved over a billion years to bind wall targets that cannot easily mutate, as a phage that does not produce a functional lysin would be trapped inside the infected host and thus lost from the population (42). Supporting this, resistance to phage lysins was not observed following selection procedures that readily produce antibiotic resistance (37, 43). Another advantage of targeting wall carbohydrates is that they are often conserved across species, resulting in a broad range of target organisms. For example, AtlA lysibody and ClyS lysibody bound all strains of *S. aureus* tested as well as several coagulase-negative staphylococci, whereas PlySs2 lysibody bound streptococci and enterococci in addition to staphylococci. The protein targets of monoclonal antibodies, on the other hand, are often variable even at the species level and may not always be expressed (44–47). This may limit the strain coverage of certain monoclonal antibodies and could also result in the selection of escape mutants during the course of treatment.

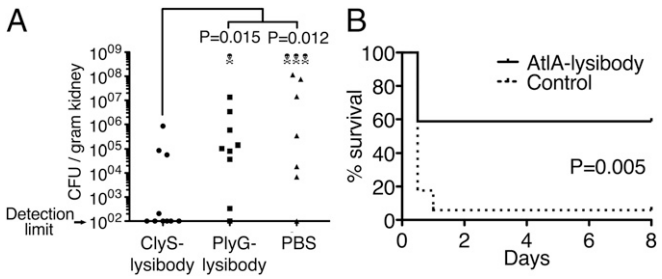

Fig. 5. Lysibodies protect mice from MRSA infection in kidney abscess and bacteremia models. (*A*) Five-week-old female BALB/C mice were injected i.p. with 1 mg of the *S. aureus*-specific ClyS lysibody, *B. anthracis*-specific PlyG lysibody, or PBS. One day later, the mice were injected i.p. with 2.5×10^6 *S. aureus* USA600 (methicillin-resistant, vancomycin-intermediate) in 5% mucin. Mouse viability was monitored daily for 4 d, at which time the mice were euthanized, and the bacterial load per gram in the kidneys was determined through homogenization, serial dilution, and plating. Aggregate data from four experiments are presented ($n = 10$ in each group). Statistical significance was determined using two-tailed Mann–Whitney test. (*B*) Five-week-old female BALB/C mice were injected i.p. with 0.3 mg AtlA lysibody or PBS ($n = 17$ in each group). One day later, mice were injected i.p. with 2×10^6 *S. aureus* MW2 (USA400, methicillin-resistant) in 5% mucin. Mouse viability was monitored for 8 d. Data represent aggregate results from four experiments. Statistical significance was determined using the Gehan–Breslow–Wilcoxon test.

A potential drawback associated with frequent/repeated lysibody treatment could be that part of the lysibody molecule is foreign to humans and may therefore elicit an immune response, the effects of which are currently unknown. However, reduction of binding domain immunogenicity could be achieved through reduction of T effector epitopes and introduction of Treg epitopes (48). Furthermore, development of antilysibody antibodies in a treated patient would likely only occur after the infecting organism has already been eliminated. The intended clinical use of lysibodies is to treat life-threatening infections, either alone or in combination with conventional therapy to help boost the opsonic response to the infecting organism. For many individuals, such life-threatening infections are a once-in-a-lifetime event for a given pathogen, and thus, development of antilysibody antibodies would be irrelevant for these patients. In cases of repeated life-threatening episodes, however, a lysibody with a different binding domain could be used; as we have shown here, development of multiple distinct lysibodies to a single organism is quite possible.

There is an urgent clinical need to create new treatment options to staphylococcal infections (17). Other than antibiotics, to which staphylococci show increased resistance, no other anti-infective is currently available. Hospitalized patients and those undergoing immunosuppressive therapy are particularly vulnerable, as highly virulent drug-resistant bacteria have become endemic in many healthcare facilities (19). Recent technological advances in the production of recombinant antibodies have made the routine use of these molecules in the clinic increasingly feasible. Therapeutic antibodies and vaccines are now aggressively being pursued as an alternative treatment for antibiotic-resistant bacterial pathogens, as indicated by the number of such agents reaching advanced stages of clinical trials (1–3). To date, all attempts to produce an effective *S. aureus* vaccine or therapeutic antibodies have failed to protect patients in clinical trials despite showing promise in preclinical studies. Although the reason for this failure is still unclear, it has been proposed that targeting only a single surface antigen may not have been sufficient to confer protective immunity, due to the vast array of redundant virulence factors *S. aureus* possesses. Staphylococcal protein A has also been proposed as a factor preventing the development of effective immunity, by nonspecifically activating B cells, leading to the deletion of potentially protective B-cell clones (49). Many current vaccine development efforts are targeting multiple factors in an attempt to overcome some of these limitations (50). In this context, lysibodies could provide a unique solution. By targeting carbohydrate epitopes that are highly abundant, conserved, and indispensible, lysibodies may lead to a better outcome compared with previous single-target therapeutic antibodies.

In conclusion, our approach opens an avenue for the development of therapeutic antibodies, using binding domains that were optimized through evolution. Lysibodies could be produced for a range of additional Gram-positive pathogenic bacteria, given the wealth of autolysins and phage lysins found in nature (15). Lysibodies therefore represent a broad class of anti-infectives that resolve the long-standing problem of effectively targeting bacterial surface carbohydrates with antibodies. More broadly, our results strongly suggest that proteins or protein domains with high-affinity binding for the surface of a bacterium, virus, or parasite may be Fc-modified to produce a functional opsonic antibody.

Methods

Ethics Statement. Procedures involving human subjects were approved by the Rockefeller University Institutional Review Board (IRB number VFI-0790).

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Informed consent was obtained from all subjects. Mouse work was approved by and mice were cared for in accordance with the Rockefeller University Institutional Animal Care and Use Committee (protocol 14691H) (51).

Additional Methods. A list of primers used in this study is provided in Table S1. A list of strains used in this study is provided in Table S2. Additional methods are provided in *SI Methods*.

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