Targeted Nasal Vaccination Provides Antibody-Independent Protection Against *Staphylococcus aureus*

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Despite showing promise in preclinical models, anti–*Staphylococcus aureus* vaccines have failed in clinical trials. To date, approaches have focused on neutralizing/opsonizing antibodies; however, vaccines exclusively inducing cellular immunity have not been studied to formally test whether a cellular-only response can protect against infection. We demonstrate that nasal vaccination with targeted nanoparticles loaded with *Staphylococcus aureus* antigen protects against acute systemic *S. aureus* infection in the absence of any antigen-specific antibodies. These findings can help inform future developments in staphylococcal vaccine development and studies into the requirements for protective immunity against *S. aureus*.

Keywords. vaccine; *Staphylococcus aureus*; cellular immunity; mucosal; adjuvant; nanoparticle.

Infections caused by antibiotic-resistant *Staphylococcus aureus* are causing a global epidemic and present an urgent and unmet need for effective vaccines [1]. Although a number of vaccines were effective in preclinical challenge models, these subsequently failed in clinical trials [2] due at least in part to a lack of insight into what constitutes protective immunity in the human host. Until recently, anti–*S. aureus* vaccine approaches have focused on the induction of neutralizing/opsonizing antibodies, but there is increasing evidence that cellular immunity may be equally or more important for protective immunity [3]. Indeed, vaccines expanding T-helper type 1 (Th1) and T-helper type 17 (Th17) cells conferred protection in murine models of *S. aureus* infection [4]. However, to date, systems that exclusively induce cellular immunity in the absence of humoral immunity have not been studied to determine if a cellular-only response can protect against systemic staphylococcal infection.

Because mucosal vaccination can potentially stimulate an immune response both at the mucosae and systemically, and has the advantage of being needle-free, our objective was to design a mucosal *S. aureus* vaccine that selectively promoted cellular immunity. Initiating an immune response at the mucosae is hindered by several factors, particularly poor uptake across epithelial barriers. Targeting mucosal antigen-sampling microfold (M) cells is a promising approach to address inefficient transepithelial vaccine uptake [5]. *Ulex europaeus* agglutinin I (UEA-1), a fucose binding lectin from gorse, binds to [6] and can enhance the transcytosis of nanoparticles across M cells [7], potentially delivering the attached antigen to subepithelial dendritic cells. Thus, we investigated the potential of targeting particulate vaccines to M cells with UEA-1 and a UEA-1 peptidomimetic (UEA-1m) [8].

METHODS

Animals

Female BALB/c and C57BL/6 mice were obtained from Harlan Laboratories and Charles River Laboratories and were used at 8–16 weeks of age. Animals were maintained in a specific pathogen-free environment at the TCD Bioresource facility. All mice were maintained according to European Union regulations, and experiments were performed under license from the Irish Department of Health and Children and with approval from the Trinity College Dublin Bioresources Ethics Committee.

Materials

Streptavidin-coated polystyrene nanoparticles (300–390 nm) were supplied by Spherotech Inc and Corpuscular Inc.

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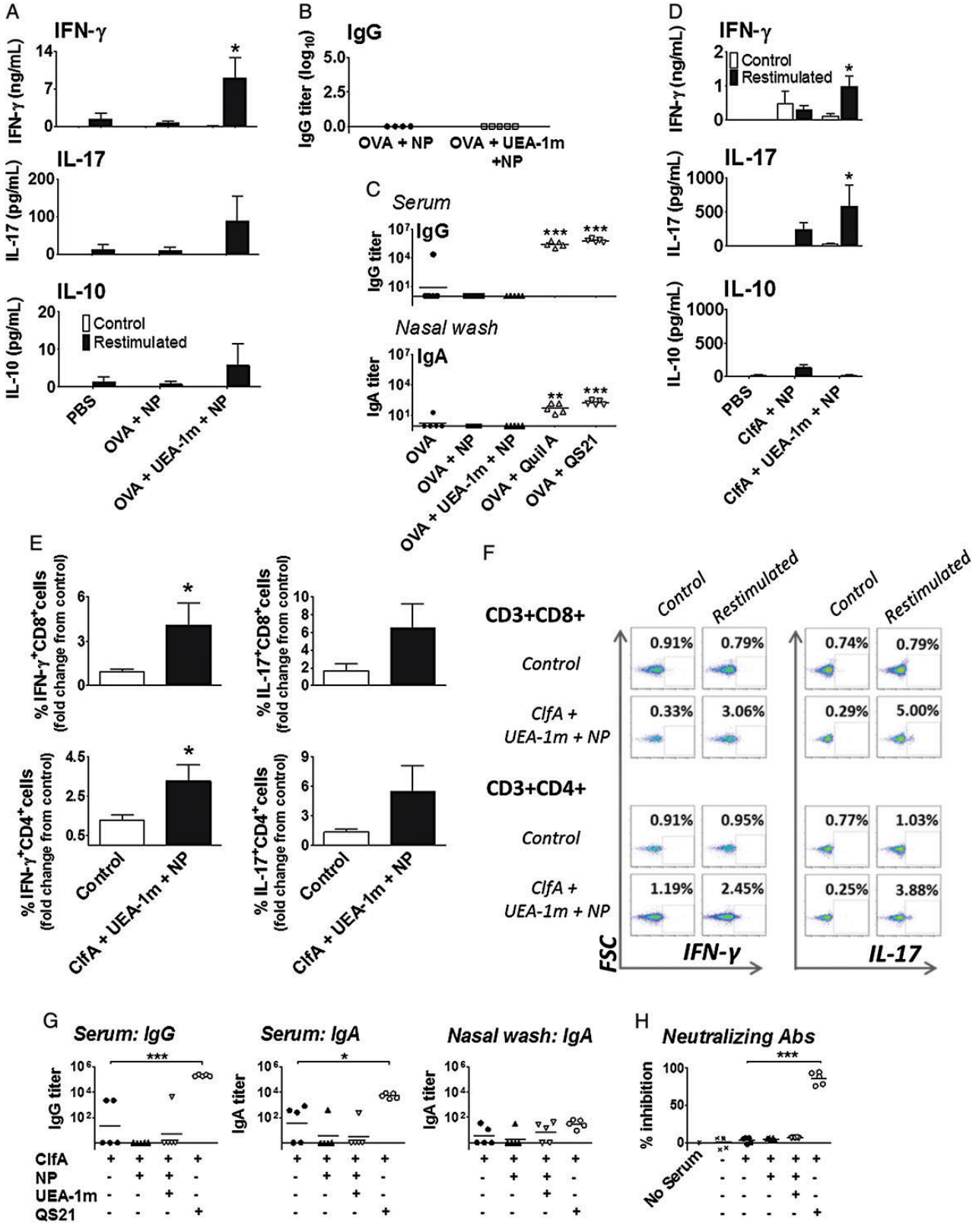

Figure 1. Lectin-targeted mucosal delivery of nanoparticulate antigens induces strong T-cell responses in the absence of a humoral response. Mice were immunized intranasally on days 1, 14, and 28 with phosphate-buffered saline (PBS) only as a control or ovalbumin (OVA; 10 μg) with or without *Ulex europaeus* agglutinin I peptidomimetic (UEA-1m; 10 μg), attached to nanoparticles (NP). *A*, Antigen-specific responses by cells isolated from the cervical lymph nodes were determined on day 35, by ex vivo restimulation with OVA (500 μg/mL) for 72 hours, and subsequent enzyme-linked immunosorbent assay (ELISA) to determine cytokine production. Mean ± SEM. **P* < .05 vs nontargeted particles; 1-way analysis of variance (ANOVA) and Tukey posttest

Biotinylated UEA-1m (synthesized by Polypeptide), and ovalbumin (OVA) antigen (Sigma) were both provided as lyophilized powders and reconstituted in endotoxin-free phosphate-buffered saline (PBS). The A domain of clumping factor A (ClfA; amino acids 40–559) was expressed as a hexahistidine-tagged recombinant protein and purified by Ni^{2+} chelate chromatography. Both OVA and ClfA antigens were biotinylated with the EZ-Link Sulfo-NHS-LC biotinylation kit (Thermo Scientific). Cytokine and antibody enzyme-linked immunosorbent assay (ELISA) detection kits were obtained from R&D Systems, BD Pharmingen, and BioLegend. Complete RPMI (cRPMI) comprised RPMI 1640 (BioSera), 10% fetal calf serum (BioSera), 1% penicillin-streptomycin (Invitrogen), and 1% L-glutamine (Invitrogen). Fluorescently conjugated antibodies for flow cytometry were purchased from BD Biosciences (anti–interleukin 17A [IL-17A] PerCP-Cy 5 clone TC11-18H10) and eBiosciences (anti–interferon [IFN]–γ PE Cy7 clone XMG 1.2). Quil A saponin was obtained from Brenntag, and the fractionated derivative of Quil A (QS21) was provided by the Vaccine Formulation Laboratory in Lausanne, Switzerland.

Vaccine Formulations

Biotinylated antigen (OVA or ClfA) and biotinylated UEA-1m were incubated with streptavidin-coated polystyrene nanoparticles for 1 hour at room temperature in sodium phosphate buffer (pH 5.5). Fifty micrograms of OVA was administered by oral vaccination, but 10 μg OVA and 2 μg ClfA was sufficient for intranasal vaccine formulations (adequate quantity to induce a cellular response to targeted nanoparticles). UEA-1m was incorporated at 10 μg per vaccine formulation (attached to nanoparticles). Protein attachment was determined by bicinchoninic assay (Thermo Scientific) of the supernatant, and the particles were resuspended in PBS (pH 7.4) before use. The contents of each formulation are detailed in the Figure legends.

Immunization Strategies

Groups of mice (n = 5) were immunized orally on 3 consecutive days (days 0, 1, and 2) and boosted 3 weeks later (days 21, 22, and 23) with PBS only, OVA (50 μg) nanoparticles, or OVA (50 μg) + UEA-1m (50 μg) nanoparticles. Mice were immunized intranasally on days 0, 14, and 28 by dropping the vaccine formulation (20 μL maximum volume containing OVA or ClfA antigen in solution or particulate form) onto each nostril and allowing the animal to inhale the vaccine. Group sizes for mucosal vaccinations and challenge were determined based on our previous experience and published reports [9, 10]. Blood samples were taken from the tail vein before each immunization for analysis of antibody production. Tissues were isolated after killing, the cells isolated by mechanical disruption, and restimulated with antigen in vitro.

Measuring the Cellular and Humoral Response to Antigen

Antigen-specific antibody titers in the serum and nasal washes, and cytokine secretion by restimulated lymphocytes (cultured for 72 hours) were determined by sandwich ELISA as described previously [9]. All samples were analyzed in triplicate. Cytokine production by $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells was detected by restimulating the cells for 6 hours in vitro in cRPMI with or without antigen. Brefeldin A (10 μg/mL; Sigma) was added to the culture after 1 hour to prevent cytokine export from the cells. After 6 hours the T cells were labeled with anti-CD3, anti-CD4, and anti-CD8 fluorophore-conjugated antibodies. Following fixation and permeabilization with FIX&PERM (ADG Bioresearch GmbH), intracellular IFN-γ and IL-17 were labeled. Data were acquired with a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc).

Measuring Neutralizing Antibodies in Serum

The presence of neutralizing antibodies was determined by testing the ability of serum from immunized mice to inhibit the

Figure 1 Continued. (n = 5). *B*, Serum samples harvested from blood taken on day 35 were analyzed for the presence of OVA-specific immunoglobulin G (IgG). Each data point represents an individual animal. *C*, Nasal delivery of QS21-adjuvanted OVA promotes humoral immunity. Mice were immunized intranasally on days 1, 14, and 28 with PBS or OVA (10 μg) either in solution or conjugated to nanoparticles with UEA-1m (10 μg), or with OVA and Quil A saponin or a purified derivative of Quil A (QS21), both at 10 μg per dose. Blood and nasal cavity washes were taken on day 35. Antigen (OVA)–specific antibody titers were determined by ELISA. Each data point represents an individual animal (n = 5); the black bars denote the mean. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs antigen alone; 1-way ANOVA and Tukey posttest (n = 5). *D–H*, Nasal immunization with *Staphylococcus aureus* clumping factor A (ClfA). Female C57BL/6 mice were immunized intranasally on days 1, 14, and 28 with ClfA (2 μg) attached to nanoparticles alone or targeted with UEA-1m (10 μg). Antigen-specific responses by cells isolated from the spleen were determined on day 35, by ex vivo restimulation with ClfA (10 μg/mL), and subsequent ELISA (*D*) and intracellular staining (*E* and *F*) to determine cytokine production. Antigen-specific responses by splenic $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells were assessed by ex vivo restimulation of the cells with ClfA for 6 hours, and subsequent flow cytometric analysis of interferon (IFN)-γ and interleukin 17 (IL-17)–producing cells. Representative dotplots showing the percentage of IFN-γ or IL-17–producing T cells. Unstimulated cells are on the left of each panel, ClfA-stimulated cells on the right. Pooled data showing the fold change in percentage of cytokine-producing T cells in the spleen (compared to unstimulated control). *G*, Anti-ClfA IgG antibody titers were assessed by ELISA in day 35 serum and nasal wash samples. The presence of neutralizing antibodies was determined by measuring the ability of serum to inhibit ClfA-mediated *S. aureus* adherence to fibrinogen. *Staphylococcus aureus* was preincubated with serum, and adherence to fibrinogen was calculated as a percentage of values measured in control wells lacking serum. Data are representative of 2–3 independent experiments. Each data point represents an individual animal; the black bars denote the mean. $^{***}P < .001$ by 1-way ANOVA and Tukey posttest (n = 5).

adherence of *S. aureus* to fibrinogen. Microtiter plates were coated with fibrinogen (2 μg/mL) overnight at 4°C and blocked for 2 hours at 37°C with 5% (w/v) bovine serum albumin. *Staphylococcus aureus* PS80 was grown to stationary phase, washed, and incubated with mouse serum (1:60 dilution) for 30 minutes at room temperature before being added to the wells of a fibrinogen-coated plate and incubated for 1.5 hours at 37°C. After washing with PBS, adherent cells were fixed with formaldehyde (25% v/v) and stained with crystal violet, and the A_{570} nm was measured. Adherence was expressed as a percentage of bacterial adherence in the absence of serum, and percentage of inhibition was determined by subtracting the percentage adherence values from 100.

Staphylococcus aureus Infection

Staphylococcus aureus PS80 has been previously described [10]. Staphylococci were cultivated from frozen stocks on Columbia agar with 2% NaCl. Bacteria were resuspended to an appropriate concentration in PBS. Nasally immunized C57BL/6 mice were infected with a sublethal inoculum of *S. aureus* (5×10^8 colony-forming units [CFU]) by intraperitoneal injection, on day 36, and were sacrificed 3, 24, and 72 hours postinfection (5 mice per treatment group, per timepoint; group size was determined through optimization of the challenge model [10]). At specific timepoints postchallenge, the peritoneal cavity was lavaged with sterile PBS and the kidneys and spleens were harvested and homogenized in PBS. Serial dilutions of the peritoneal exudates and organ homogenates were prepared and plated onto tryptic soy agar to determine the numbers of *S. aureus* CFU. Serum was collected from each mouse 1 day prior to each vaccination for analysis of antibody titers. To quantify phagocyte infiltration, total leukocytes isolated from the peritoneal exudates were enumerated using a hemocytometer and were then stained with monoclonal antibodies against neutrophil ($CD11b^+Ly6G^+F4/80^-$) and macrophage ($CD11b^+Ly6G^-F4/80^+$) surface markers before analysis by flow cytometry (BD FACSCalibur).

Statistical Analysis

Data are presented as mean (±SEM) from 5 mice per experimental group, tested individually in triplicate. $P < .05$, $P < .01$, and $P < .001$ denote statistical significance between groups by 1-way analysis of variance and Tukey posttest, or by 1-tailed unpaired Student *t* test, as specified in the Figure legends.

RESULTS AND DISCUSSION

Nasal vaccination with UEA-1m–targeted, antigen (OVA)–loaded nanoparticles (300–390 nm diameter) promoted enhanced antigen-specific T-cell responses compared to antigen conjugated to nontargeted particles (Figure 1*A*). The most striking finding was the Th1- and Th17-biased response elicited by targeted nanoparticles (Figure 1*A*), without the induction of any detectable antigen-specific serum immunoglobulin G (IgG) (Figure 1*B*). Similarly, oral vaccination with UEA-1m–targeted nanoparticles enhanced antigen-specific cellular responses (Supplementary Figure 1), compared to untargeted nanoparticles in the absence of a detectable antibody response. In contrast, antibodies were readily induced both systemically (serum IgG) and locally (nasal wash immunoglobulin A [IgA]) following intranasal administration of OVA in combination with Quil A, a potent saponin adjuvant [11], or QS21 (Figure 1*C*), an adjuvant active fraction of Quil A that displays reduced toxicity [11]. Our findings support a report that UEA-1 targeting of a nasal human immunodeficiency virus vaccine elicited enhanced cellular responses [12] but differs in that our system did not induce any local or systemic humoral immunity.

Having identified a mucosal adjuvant formulation that selectively promoted T-cell responses, we generated a model anti–*S. aureus* vaccine, based on UEA-1m–targeted nanoparticles coupled with the *S. aureus* surface antigen ClfA, and investigated its efficacy in protection against systemic *S. aureus* infection. ClfA is an important *S. aureus* virulence factor, which promotes bacterial adherence to fibrinogen [13]. Whereas immunization of mice with recombinant ClfA A domain formulated with Freund's complete adjuvant induced protective humoral immunity [13], protection was IL-17A–dependent following injection of the antigen in alum adjuvant [14]. These reports highlight the promise of this ubiquitously expressed staphylococcal surface protein as a candidate antigen for the induction of protective cell-mediated immunity. In line with this, a vaccine based on a *Candida albicans* surface protein with structural similarity to ClfA [15] induced protective anti–*S. aureus* immunity that was critically dependent on the induction of Th1 and Th17 responses [4].

Initially, we investigated whether UEA-1m–targeted ClfA-loaded nanoparticles could promote antigen-specific T-cell responses in the absence of antibodies. As in the case of OVA, UEA-1m targeting significantly enhanced ClfA-specific IFN-γ and IL-17 production (Figure 1*D*). Splenic $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells from targeted nanoparticle-vaccinated mice produced IFN-γ and IL-17 upon in vitro restimulation with ClfA, as reflected in an increased percentage of antigen-specific cytokine-producing T cells and a greater fold increase in cytokine-producing T cells vs controls (Figure 1*E, F*). Anti-ClfA antibodies were undetectable in serum samples from animals vaccinated with ClfA-loaded, UEA-1m–targeted nanoparticles, whereas high titers of antigen-specific serum IgG, IgA (Figure 1*G*), and function-neutralizing antibodies capable of blocking the adherence of ClfA-expressing bacteria to fibrinogen were elicited by vaccination with ClfA and QS21 (Figure 1*H*).

Having established the ability of the ClfA-loaded, UEA-1m–targeted nanoparticles to elicit ClfA-specific cellular immune responses in the complete absence of any specific humoral

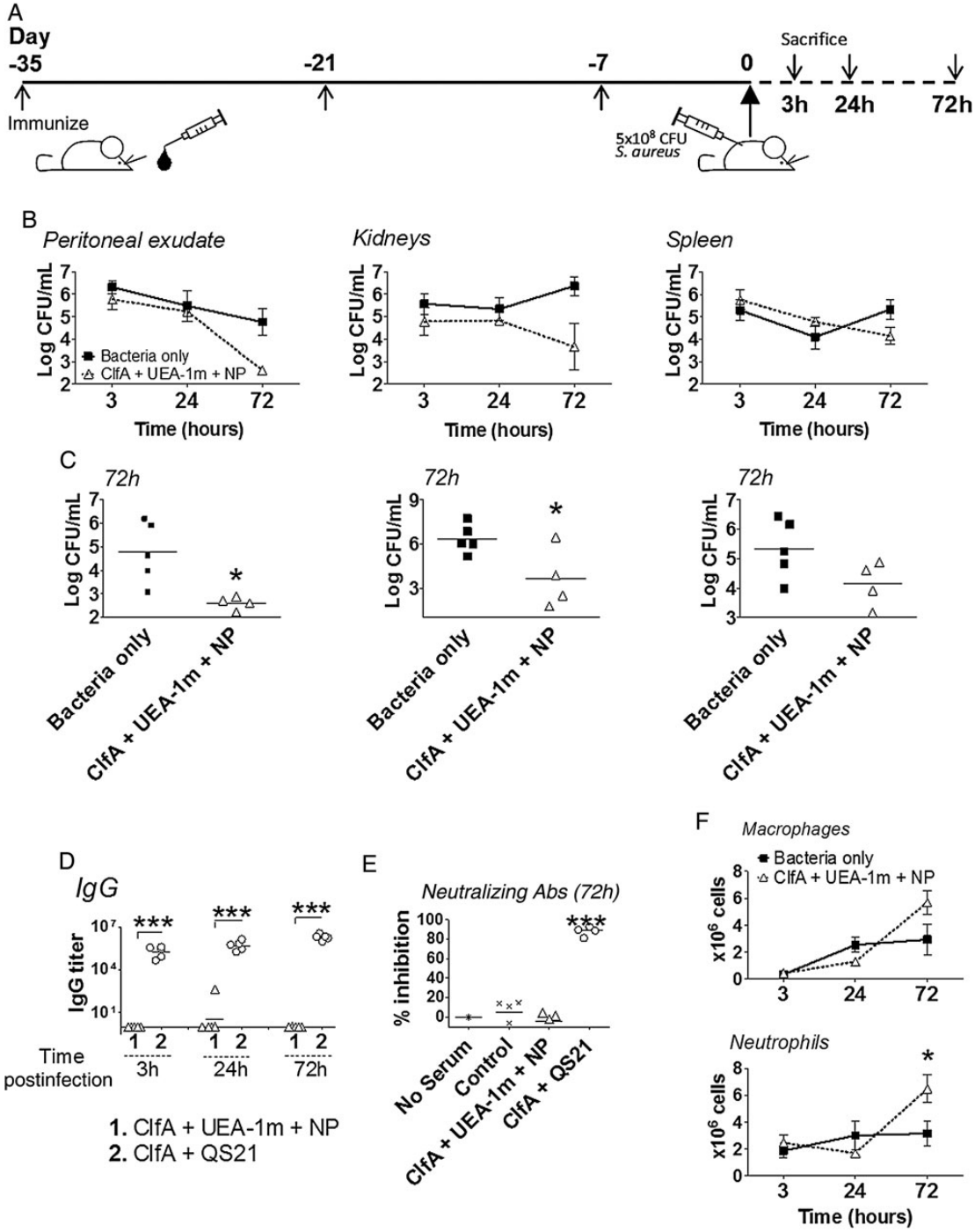

Figure 2. The induction of cellular immunity by a nasal targeted nanoparticulate *Staphylococcus aureus* vaccine is sufficient for clearance of a systemic *S. aureus* infection. *A*, Immunization strategy. Mice were immunized intranasally on days 1, 14, and 28 with phosphate-buffered saline (PBS) or with clumping factor A (ClfA; 2 μg) and *Ulex europaeus* agglutinin I peptidomimetic (UEA-1m; 10 μg) attached to nanoparticles (NP). Mice were sacrificed at time 0 (day 35, before infection), and at 3, 24, and 72 hours after intraperitoneal infection with 5×10^8 colony-forming units (CFU) *S. aureus* (strain PS80). Data are representative of 2 independent experiments. *B* and *C*, Bacterial counts. The peritoneal exudate, kidneys, and spleen were harvested at each time point, homogenized, and cultured, and CFUs were determined. CFU burden is expressed as the mean ± SEM over time (n = 5, top panel), and in each tissue at 72 hours postinfection. *C*, Statistical significance between groups is denoted by $^*P < .05$ and $^{**}P < .01$ (1-tailed unpaired Student *t* test). *D* and *E*, Antibody responses following challenge. Anti-ClfA immunoglobulin G (IgG) antibody titers were determined by enzyme-linked immunosorbent assay on serum samples recovered at each timepoint postinfection. The presence of neutralizing antibodies was determined by measuring the ability of serum to inhibit ClfA-mediated *S. aureus* adherence to fibrinogen. *Staphylococcus aureus* was preincubated with serum, and adherence to fibrinogen was calculated as a percentage of values measured in control wells lacking serum. Each data point represents an individual animal (n = 5); the black bars denote the mean. $^{***}P < .001$ by 1-way analysis of variance and Tukey posttest. *F*, Phagocytes at the site of infection. The total numbers of peritoneal macrophages and neutrophils in control and vaccinated mice were determined by flow cytometry at 3, 24, and 72 hours following infection. $^*P < .05$ by 1-tailed unpaired Student *t* test (n = 5).

immunity, we next determined the ability of this vaccine to induce protective immunity against systemic *S. aureus* infection. Groups of mice were nasally immunized with either ClfA on UEA-1m–targeted nanoparticles or ClfA with QS21 prior to intraperitoneal challenge with a sublethal dose of *S. aureus* (Figure 2*A*). Bacterial load both locally at the site of infection (peritoneal cavity) and systemically (kidneys and spleen) was reduced in both vaccinated groups over the course of infection (Figure 2*B*, Supplementary Figure 2), with a significant (approximately 2 log) reduction in systemic bacterial dissemination to the kidneys at 72 hours postinfection, in animals receiving either vaccine compared to unvaccinated control animals (Figure 2*C*, Supplementary Figure 2).

Antigen-specific serum antibody titers induced by the QS21-adjuvanted vaccine remained stable over the course of immunization, and neutralizing antibodies were still present 72 hours postinfection but were completely undetectable in animals receiving the UEA-1m–targeted nanoparticle vaccine (Figure 2*D* and *E*). In addition to promoting T-cell responses (Figure 1*D*), immunization with ClfA on UEA-1m–targeted nanoparticles also increased local infiltration of neutrophils and macrophages to the peritoneal cavity (Figure 2*F*) at 72 hours postinfection compared to unvaccinated animals. In contrast, there was no significant increase in phagocyte infiltration to the peritoneal cavity in animals that received the QS21-adjuvanted vaccine (Supplementary Figure 2*C*).

Remarkably, these data demonstrate that a targeted nasal vaccine promotes clearance of an acute *S. aureus* systemic infection, but also that a purely cellular response is sufficient for this protection. This is the first formal demonstration that vaccine-induced *S. aureus* antigen-specific cellular immunity in the absence of detectable antibody responses can protect against staphylococcal infection and expands upon previously published work indicating the importance of cellular immunity in vaccine-induced protection against *S. aureus* infection [4, 15]. Our targeted mucosal vaccine approach will prove a valuable tool not only in dissecting protective immunity to *S. aureus* but also for other bacterial infections where Th1 and Th17 responses have been shown to contribute to protective immunity, including *Bordetella pertussis* and *Streptococcus pneumoniae*.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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