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Epitope-Based Immunization Elicits High-Affinity Antibody Responses to the Immunologically Subdominant D3 Epitope of *Staphylococcus aureus* Coproporphyrinogen III Oxidase

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Abstract

Background: *Staphylococcus aureus* (*S. aureus*) remains a major global pathogen causing a wide spectrum of diseases. Human adults harbor circulating antibodies against most staphylococcal antigens, which provide little protection against invasive *S. aureus* infection. Conventional *S. aureus* vaccines are poised to predominantly repeat the natural, non-protective antibody responses. **Objective**: We investigated the immunological feasibility of an epitope-focused vaccine targeting the D3 epitope of coproporphyrinogen III oxidase (CgoX), which is recognized by the protective monoclonal antibody anti-CgoX-D3. **Methods**: The potential of the D3 epitope to break the dominant but non-protective immunity to CgoX was investigated in laboratory mice. The seroprevalence of antibodies against the D3 epitope was investigated in sera from healthy blood donors and patients with *S. aureus* bacteremia. **Results**: All sera from 204 healthy blood donors harbored antibodies against CgoX, whereas only 10.3% contained antibodies against the D3 epitope, indicating its immunological subdominance. In patients with invasive *S. aureus* infection, D3-specific responses were more frequent but remained at low serum concentrations, confirming the quantitative subdominance of the protective D3 epitope. Prime-boosting experiments in mice with combinations of whole CgoX and a CRM197-CgoX-D3 conjugate (D3-CRM) revealed that mice primed and boosted with whole CgoX developed weak D3-specific responses. Boosting CgoX-primed mice with D3-CRM resulted in the generation of robust titers of high-affinity D3 binding antibodies. **Conclusions**: The immunization history with CgoX markedly influences subsequent D3-specific antibody responses. Epitope-focused vaccination with D3-CRM can overcome the attenuation associated with prior CgoX immunization and elicit high-affinity D3-specific antibodies.

Keywords: *Staphylococcus aureus*; subdominant epitope; immunological imprint; epitope-specific vaccination; coproporphyrinogen III oxidase; pre-existing immunity

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1. Introduction

Staphylococcus aureus (*S. aureus*) remains a major global health threat, causing a broad spectrum of diseases ranging from mild skin infections to fulminant sepsis, pneumonia, osteomyelitis, and endocarditis [1]. The increasing prevalence of multidrug-resistant strains, particularly methicillin-resistant *S. aureus* (MRSA), has further elevated the urgency of effective preventive strategies [2,3]. Despite decades of research, however, no vaccine has yet provided durable protection in humans [4–8]. Traditional approaches, including whole-cell vaccines, toxoid formulations, and recombinant protein subunits, have repeatedly failed in advanced clinical trials, underscoring the pathogen's exceptional immune-evasion capacity and the difficulty of defining a clear correlate of protection [4,5,9].

Multiple factors likely contribute to these failures. *S. aureus* expresses a vast arsenal of virulence factors with substantial functional redundancy, enabling it to evade both innate and adaptive immunity. Humans also lack effective natural immunity. Because nasal colonization is common, most adults harbor circulating antibodies against numerous staphylococcal antigens; however, these antibodies provide little protection against invasive *S. aureus* infection [10,11]. Thus, induction of a high-titer antibody response is not equivalent to protection.

Preclinical and clinical data repeatedly indicated that although immunization with *S. aureus* antigens usually results in high antibody titers, this does not confer protection against *S. aureus* infection [8]. Clearly, induction of a high-titer anti-*S. aureus* antibody response by a vaccine is not tantamount to protection. Although colonization is common, naturally acquired antibodies do not confer protection against invasive infection. Indeed, high-titer antibody responses elicited by classical vaccines have not translated into clinical benefit, suggesting that antibody quantity alone is an inadequate marker of protection. In some cases, non-protective or even detrimental immune responses may be induced [4,5,12,13], echoing principles known from viral vaccine research [14]. These challenges highlight the need to distinguish protective from non-protective—or potentially disease-enhancing—B-cell epitopes, and to design vaccines that selectively focus the immune system on epitopes with demonstrated protective potential.

Epitope-based vaccines have thus emerged as a promising next-generation strategy [15,16]. By restricting the immune response to well-characterized B- or T-cell epitopes, these vaccines can enhance immunogenic precision while minimizing off-target and potentially counterproductive immune activation. Advances in reverse vaccinology, structural immunology, and functional mapping using monoclonal antibodies (mAbs) now enable the identification of epitopes that are necessary and sufficient for protection [15].

As for *S. aureus*, several epitope-centered candidates have shown protective efficacy in animal models. Protective linear or conformational epitopes have been mapped in antigens such as alpha-toxin, iron-regulated surface determinant B (IsdB), manganese transport protein C (MntC), glyceraldehyde-3-phosphate dehydrogenase C (GapC), fibronectin-binding protein A (FnBPA), and coproporphyrinogen III oxidase (CgoX), sometimes with demonstrable opsonophagocytic killing or robust Th1/Th17 activation [17–22].

One major obstacle to vaccination against subdominant epitopes is immune imprinting by immunodominant antigens. Any further exposure to the same antigen results in a recall of this imprint, favoring recall responses to immunodominant epitopes, while suppressing the development of immunity to subdominant antigens. Non-protective immune imprints as a result of repeated and long-time exposure to immunodominant *S. aureus* antigens interfere with subdominant but potentially protective determinants [12,23,24]. *S. aureus*-associated immune imprinting is not expected in laboratory mice, because laboratory mice are kept under pathogen-free conditions and, therefore, are not exposed to *S. aureus*. With regard to *S. aureus*, immunogenicity of a subdominant epitope in specific-pathogen-free

mice may not predict immunogenicity in humans, who typically carry extensive pre-existing anti-staphylococcal immune imprints.

Accumulating evidence from other major human pathogens suggests that subdominant antigens are promising targets for protective immunization. For example, the subdominance of the hypervariable region (HVR) of the M protein of *Streptococcus pyogenes* suggests that this domain is indispensable for virulence and that evolution has favored the appearance of mechanisms that allow escape from antibody attack on the HVR [25]. While the HVR is subdominant when present in an intact M protein, peptides derived from HVRs elicit good antibody responses [26]. The amino-terminal domains of the repetitive immunogenic bacterial protein (Rib) and alpha proteins from *Streptococcus agalactiae* are subdominant when present in the intact proteins; however, a fusion protein derived from these domains was immunogenic even without adjuvants, and elicited good protective immunity [27]. Thus, the subdominant amino-terminal regions of Rib and alpha protein are targeted by protective antibodies and are of interest for vaccine development. Hemagglutinin (HA) of influenza virus contains many subdominant sites such as the HA stem as targets for protective antibodies. The immunodominant head of influenza virus HA elicits strain-specific protective antibodies, while the subdominant stem elicits broadly protective antibodies. The various methods that focus antibody responses on subdominant HA sites now offer much hope for the development of a universal influenza vaccine [28].

In *S. aureus*, epitopes identified through human immunological studies such as those revealed in clinical testing of multicomponent subunit vaccines, such as the recombinant five-antigen *S. aureus* vaccine (rFSAV) [17,19], provide growing evidence that epitope-specific immunity is achievable and relevant in humans. The general obstacle in *S. aureus* vaccine development is the high prevalence of pre-existing humoral and cellular immunity [10,11]. Most individuals encounter *S. aureus* early in life, resulting in broad antibody repertoires against hundreds of staphylococcal antigens. These antibodies, however, are largely non-protective and may even compete with or mask epitopes needed for protective responses [8]. Thus, any successful *S. aureus* vaccine must overcome the immunodominance of non-protective antigens and instead redirect immunity toward rarely recognized, protective epitopes [16].

We previously identified CgoX as a vaccine candidate capable of inducing protective immunity in murine *S. aureus* infection models [29]. While most mAbs raised against CgoX proved non-protective in animal studies, the antibody anti-CgoX-D3 proved to be protective as a stand-alone therapy in a septic *S. aureus* mouse model [22]. Anti-CgoX-D3 recognizes the D3 epitope, a linear 12-amino-acid sequence, which when chemically conjugated to the carrier protein BSA induces strong and protective immunity against *S. aureus* in murine infection models [22]. The D3 epitope is highly conserved among clinical *S. aureus* isolates, suggesting broad applicability.

In this study, we address key translational steps for the CgoX-D3 epitope vaccine concept. We first evaluate baseline seroprevalence of anti-CgoX and anti-CgoX-D3 antibodies in human donors and elaborate the immuno-subdominant nature of the D3 epitope. A prime–boost immunization schedule was tested and optimized using full-length recombinant CgoX and an adjuvanted CgoX-D3–CRM197 conjugate (D3-CRM). The resulting humoral response was assessed quantitatively and qualitatively by measuring antibody concentration and binding affinity to the D3 epitope.

2. Materials and Methods

2.1. Ethics Statement

Experiments with mice were performed in accordance with the Animal Protection Law of Germany in compliance with the Ethics Committee at the University of Cologne. In accor-

dance with these rules, our study protocols received ethical approval by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection–Veterinary Section, Recklinghausen, Germany, under the registration number AZ 81-02.04.2020.A163.

Serum samples from healthy volunteers were provided from the transfusion medicine department of the University Hospital Cologne, where written informed consent is obtained from all donors prior to blood donation. The use of these samples was approved by the Ethics Committee of the Medical Faculty of the University of Cologne, Germany (registration number: 21-1561), in accordance with guidelines of Good Clinical Practice (CPMP/ICH/135/95). Plasma samples from the clinical cohort were obtained under the separately approved study protocol by the Ethics Committee of the Medical Faculty of the University of Cologne (registration number: 08-160).

2.2. Recombinant Protein Purification

Expression and purification of rCgoX as C-terminally His_6-tagged protein was performed as described before [22].

LukD was cloned accordingly into pET29b vector using primer pairs listed in Table 1. $rLukD_{His6}$ was then expressed in *E. coli* BL21(DE3) and purified similarly to rCgoX by affinity chromatography using HisTrap HP columns (Cytiva) and dialyzed in 20 mM sodium phosphate buffer, pH 7.4, 0.5M NaCl. Protein concentration was determined by Pierce BCA Assay (Life Technologies GmbH, Darmstadt, Germany, cat. no. 23225) using a BSA standard curve and linear regression.

Table 1. Primer sequences for recombinant LukD cloning.

Primer Name	Primer Sequence
pET29b-LukD-NcoI-S	5′-CCATGGCTCAACATATCACAC-3′
pET29b-LukD-XhoI-AS	5′-GTGCTCGAGTACTCCAGGATTAGTT-3′

2.3. Seroprevalence Analysis of CgoX and CgoX-D3 Epitope by ELISA

Recombinant antigens CgoX and LukD were adjusted to 5 µg/mL in Bicarbonate Coating Buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and coated overnight (ON) at 4 °C with 100 µL/well in Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark, cat. no. 442404). The next day, plates were blocked for 1 h at room temperature (RT) with 200 µL/well blocking buffer consisting of TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 0.05% Tween-20; Tween-20 from AppliChem GmbH, Darmstadt, Germany, Cat. No. A4974.0500) supplemented with 5% (*w*/*v*) skim milk powder (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, Cat. No. T145.5) and 2% (*w*/*v*) bovine serum albumin (BSA; Albumin Fraction V, protease-free; Carl Roth GmbH + Co. KG, Karlsruhe, Germany, Cat. No. T844.3). Plates were washed three times with PBS-T (phosphate-buffered saline containing 0.05% Tween-20; PBS composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Human serum or plasma was diluted 1:500 in blocking buffer, applied to the plates in triplicates and incubated for 1 h at RT. After washing three times with PBS-T, plates were incubated for 1 h at RT with HRP-conjugated goat anti-human IgG (H+L) antibody (Bio-Rad Laboratories, Hercules, CA, USA, cat. no. 1721050). Plates were washed again three times with PBS-T, followed by detection with 100 µL/well TMB/E Single Reagent (Merck Millipore, Darmstadt, Germany, Cat. No. ES001-500ML) for 10 min at RT. The reaction was stopped by addition of 50 µL 2 N H_2SO_4 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany, cat. no. X873.1), and absorbance was measured at 450 nm with a Tecan plate reader (Tecan Austria GmbH, Grödig, Austria). For CgoX-specific antibody detection, the cut-off value was defined as the mean absorbance of blank wells

plus three standard deviations (mean blank OD450 + 3 SD), as no negative-control serum samples were available for this analysis.

For analysis of antibody prevalence towards the CgoX-D3 epitope, Nunc MaxiSorp™ 96-well plates were coated ON at 4 °C with 100 µL/well of streptavidin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, cat. no. S0677), diluted to 5 µg/mL in PBS. The next day, plates were washed three times with PBS-T. The synthetic lyophilized biotinylated CgoX-D3 peptide (PepScan, Lelystad, The Netherlands, order no. PEP-3700) was reconstituted in 1 mL sterile water to a stock concentration of 2 mg/mL. The stock solution was diluted to 3 µg/mL in PBS-T, and 100 µL/well was added to the plates and incubated for 2 h at RT. Subsequently, plates were blocked with 150 µL/well StartingBlock T20 (PBS) buffer (Life Technologies GmbH, Darmstadt, Germany, cat. no. 37539) for 2 h at RT. Human serum or plasma was diluted 1:50 in StartingBlock T20 buffer in triplicates and incubated at 4 °C ON. Plates were again blocked with StartingBlock T20 buffer for 2 h at RT and subsequently incubated with HRP-conjugated goat anti-human IgG (H+L) antibody (Bio-Rad Laboratories, Hercules, CA, USA, cat. no. 1721050) for 1.5 h at RT at a final dilution of 1:3000. Detection was performed using 100 µL/well TMB/E substrate for 20 min at RT and stopped by the addition of 50 µL 2 N H_2SO_4. Absorbance was measured at 450 nm with a Tecan plate reader. For CgoX-D3-epitope-specific antibody detection, the cut-off value was calculated as the mean absorbance of negative-control serum samples plus three standard deviations (mean negative-control OD450 + 3 SD).

2.4. Mouse Experiments

Female BALB/cAnNCrl mice 6–7 weeks of age were purchased from Charles River Laboratories, Sulzfeld, Germany. After arrival, mice were subsequently acclimated for at least one week and fed standard rodent diet ad libitum.

2.5. Active Immunization of Mice

Groups of 5 mice (8–9 weeks old) were immunized subcutaneously (s.c.) on days 0, 21 and 42. Animals were not randomly allocated to the immunization groups, and the animal experiments were not conducted under blinded conditions. The investigator performing the ELISA measurements was blinded to the immunization-group identities of the serum samples. On days 0, 20, 41 and 56 blood was drawn by cheek punch from the Vena facialis superficialis for serum collection in Microvette® 200 Serum Gel tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany, cat. no. 20.1291) according to the manufacturer's instructions.

For immunogenicity studies, mice were administered with either D3-CRM197 conjugate (piCHEM Forschungs- und Entwicklungs GmbH, Raaba-Grambach, Austria) or D3-BSA conjugate (Intavis Peptide Services GmbH, Tübingen, Germany) at doses of 10, 25 or 50 µg adjuvanted with alhydrogel 2% (InvivoGen, San Diego, CA, USA; cat. no. vac-alu-250).

The prime–boost studies were conducted with 100 µg recombinant CgoX, 25 µg D3-CRM197 conjugate or PBS as control, mixed with 25% alhydrogel 2%. Initial immunization and the first booster were administered 21 days apart with rCgoX and D3-CRM197 as well as combinations thereof, followed by a second booster immunization at day 42 with 25 µg D3-CRM197 for all groups, except the control.

2.6. Analysis of Mouse Antibody Titers by ELISA

For determination of antibody responses, 5 µg/mL of the respective antigen was diluted in Bicarbonate Coating Buffer and coated with 100 µL/well overnight at 4 °C in Nunc Maxisorp™ 96-well plates. Washing was performed after each step with 3x 300 µL PBS-T. Subsequent to coating, plates were blocked with 200 µL/well blocking buffer (TBS-T, 5% milk powder, 2% Albumin). Afterwards serially diluted sera were added for 1 h at

RT, followed by HRP-conjugated goat anti-mouse IgG (Sigma Aldrich, Germany, cat. No. A3673) at a final dilution of 1:3000 for 1 h at RT. Colorimetric reaction was initiated by adding TMB/E Single Reagent and stopped with 2 N H_2SO_4. Absorbance was measured at 450 nm with a Tecan plate reader.

To analyze the epitope-specific CgoX-D3 response, CgoX-D3 titers were determined as described above for human samples, except that HRP-conjugated goat anti-mouse IgG (Sigma Aldrich, Germany, cat. no. A3673) was used as secondary antibody instead.

2.7. Analysis of CgoX-D3 Mouse Serum Concentration by ELISA

For measuring standard reference-anchored concentrations of antibodies in mouse recognizing the CgoX-D3 epitope, custom-made plates were developed with JPT Peptide Technologies GmbH, Berlin, Germany, using the immobilized biotinylated CgoX-D3 peptide carrying an additional Ttds Linker on streptavidin plates. This enhanced the sensitivity of the ELISA and led to the following protocol adjustments: Custom-made plates were directly removed from the fridge and blocked with 150 µL/well StartingBlock T20 (PBS) blocking buffer. Mouse sera were serially diluted in StartingBlock T20 in duplicates and additionally purified murine moAb D3 (starting concentration 0.1 µg/mL) was serially diluted for determination of the D3-like antibody serum concentration. All incubation steps were performed for 1 h at RT. Colorimetric detection was performed with the OptEIA™ TMB Substrate Reagent Set (BD Biosciences, Franklin Lakes, NJ, USA) at RT for 10 min and stopped by the addition of 2 N H_2SO_4. Absorbance was measured at 450 nm. For comparisons of antibody concentrations between the experimental groups, fold differences in geometric mean antibody concentrations and corresponding 95% confidence intervals were calculated relative to the group primed and boosted with recombinant CgoX protein using a lognormal *t*-test (unpaired *t*-test assuming a lognormal distribution and equal geometric standard deviations between the two populations). Statistical significance was assessed using the Holm–Šídák method to correct for multiple comparisons, with an overall significance level of $\alpha = 0.05$ and performed using GraphPad Prism (Version 11.0.0).

2.8. Affinity Analysis of Anti-CgoX-D3 Specific Antibodies by ELISA

The apparent affinity of anti-CgoX-D3 antibodies was determined using the same ELISA format as described above for the antibody quantification. Mouse sera were adjusted to a CgoX-D3-specific antibody concentration of 0.1 µg/mL, as determined by the quantification ELISA. Purified murine mAb D3 was prepared at the same starting concentration (0.1 µg/mL) and all samples were serially diluted three-fold in StartingBlock T20 in duplicates. All subsequent incubation, washing, detection, and color development steps were performed as described above.

Raw absorbance values were corrected by subtraction of the blank signal prior to analysis. Apparent equilibrium dissociation constants (Kd) were determined by nonlinear regression using a one-site specific binding model in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Differences in antibody affinities between groups was determined by Kruskal–Wallis test followed by Dunn's multiple comparisons test, with *p*-values adjusted to control the false discovery rate (FDR) using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. An FDR threshold of $Q = 0.05$ was applied. Statistical analyses were performed using GraphPad Prism (Version 11.0.0).

3. Results

3.1. Seroprevalence of Natural CgoX-D3-Epitope-Specific Antibodies in Healthy Blood Donors

A challenging confounding factor for the development of a CgoX-D3-specific immunization strategy is pre-existing humoral immunity, which provides only minor protection

against invasive infection with *S. aureus*. Indeed, the failure of the *S. aureus* IsdB vaccine trial could be explained by the recall of non-protective immune imprints from prior *S. aureus* exposure [12]. Caldera et al. recently showed that pre-existing human antibodies limit the efficacy of anti-*S. aureus* monoclonal antibodies, and that such interference from imprints can be avoided by vaccinating against subdominant antigens [23]. Thus, the status of possibly pre-existing anti-CgoX and anti-CgoX-D3 specific antibodies is relevant for the assessment of the prospects of a CgoX-D3-epitope-based vaccine development.

Sera from 204 healthy blood donors at the University Hospital Cologne were analyzed for the presence of CgoX- and CgoX-D3-specific antibodies. All of the 204 healthy blood donors were found to contain antibodies recognizing full-length CgoX (Figure 1). In contrast, antibodies specifically recognizing the CgoX-D3 epitope were detected in only 10.29%. Furthermore, only one serum contained CgoX-D3-reactive antibodies at concentrations ≥ 10 µg/mL.

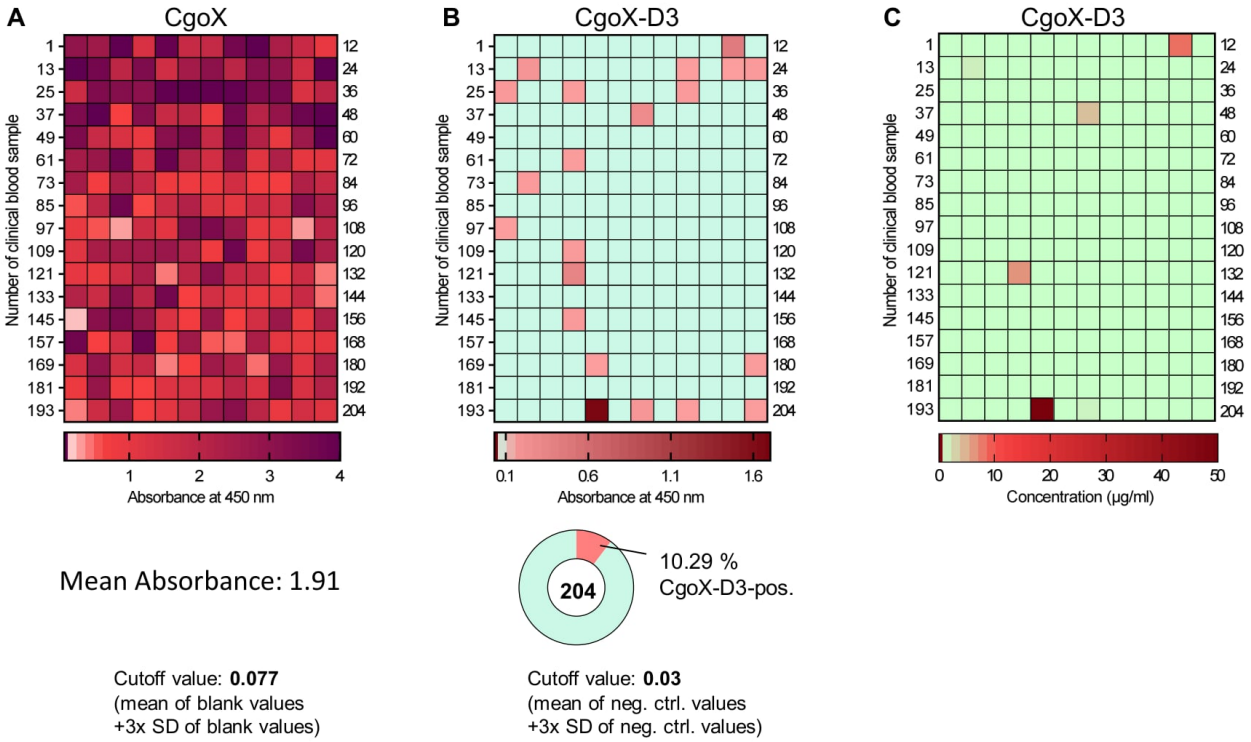


Figure 1. Seroprevalence of anti-CgoX and anti-CgoX-D3 specific antibodies in healthy blood donors. Serum samples from 204 healthy blood donors were analyzed by ELISA using full-length CgoX or the D3 epitope to test for anti-CgoX and anti-CgoX-D3 specific antibodies, respectively (**A**,**B**). Reference anti-CgoX-D3 antibody was used as standard for quantification of the respective concentration (**C**).

To address the question whether antibodies recognizing the CgoX-D3 epitope emerge in patients with ongoing invasive *S. aureus* infection, a sub-cohort of 65 (2.6%) COVID-19 patients was investigated that had microbiologically proven *S. aureus* infection and compared to a control group of 68 COVID-19 patients without ongoing *S. aureus* infection (Figure 2). COVID-19 patients with or without concomitant *S. aureus* infection showed a similar frequency of anti-CgoX-positive sera (Figure 2A,B). In contrast, the frequency of sera containing CgoX-D3-specific antibodies in COVID-19 study participants with concomitant *S. aureus* infection rose from 14.7 to 40.3% (Figure 2B). Only one serum sample showed a concentration of CgoX-D3-specific antibodies above 10 µg/mL. Likewise, antibodies recognizing the *S. aureus* leukocidin D were found in practically every blood donor and in the COVID-19 cohort at high titers (Figure A1).

These data suggest that humans rarely generate CgoX-D3-specific antibodies at large concentrations. Thus, CgoX-D3-epitope-specific immunization may generate a distinct antibody species with protective efficacy rather than adding more of the same, pre-existing but non-protective anti-*S. aureus* CgoX antibodies raised upon natural exposure to *S. aureus*.

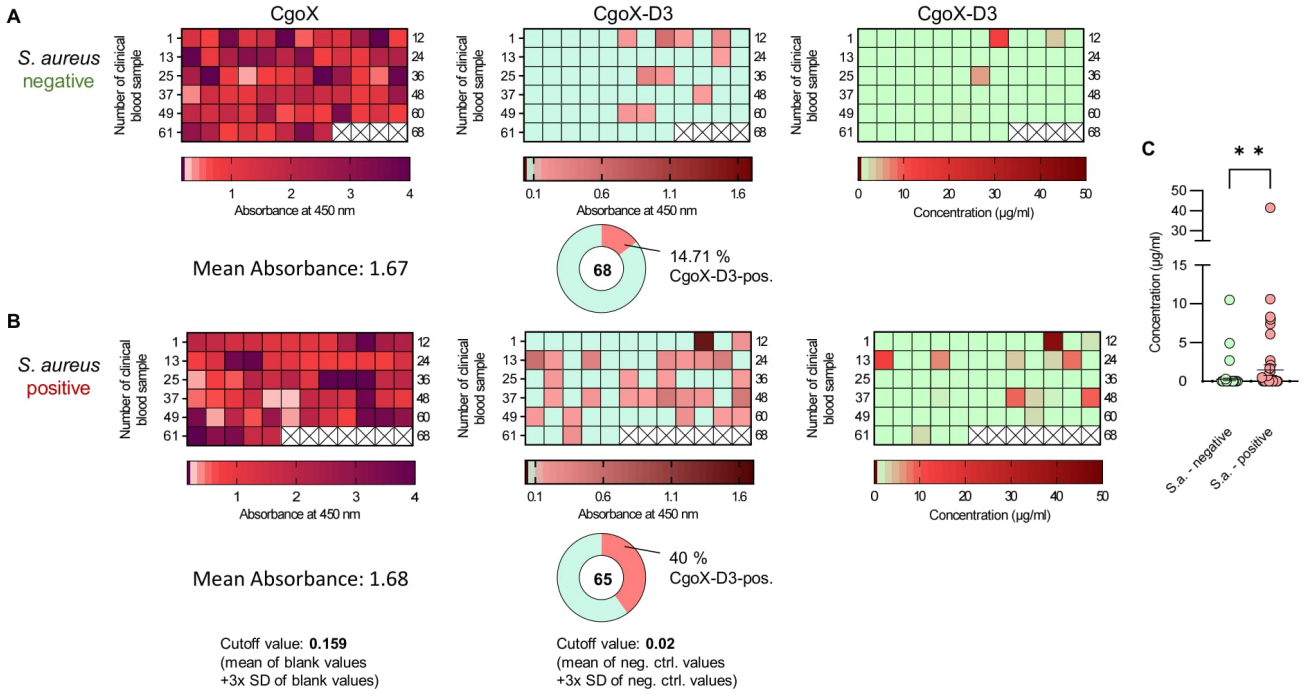


Figure 2. Seroprevalence of anti-CgoX and anti-CgoX-D3 specific antibodies in a study cohort with or without concomitant *S. aureus* infection. In total, 65 or 68 serum samples from study participants (**A**) with or (**B**) without concomitant *S. aureus* infection, respectively, were analyzed for CgoX- and CgoX-D3-specific antibodies. (**C**) CgoX-D3-specific antibody concentrations were compared using a two-tailed Mann–Whitney U test (non-parametric, unpaired test). ** indicates an exact *p*-value of 0.0074.

3.2. Immunogenicity of D3-Epitope-CRM197 Conjugates

We previously showed that the D3 epitope coupled to BSA elicited a robust antibody response that was functionally protective in a mouse sepsis model [22]. Since BSA is not a carrier protein for clinical use, we chose to conjugate the D3 epitope with the carrier protein CRM197, a nontoxic mutant of diphtheria toxin [30] that has been successfully used in many vaccines against infectious disease models [30,31]. CRM rapidly activates CD4+ T cells with a favorable Th1 and Th2 cytokine profile for activating B cells resulting in robust antibody responses [30].

The CgoX-D3-epitope-CRM197 conjugate (D3-CRM) dose-dependently elicited CgoX-D3-specific and CgoX antibodies (Figure 3A–C). To dissect whether D3-CRM-induced antibodies target the full-length CgoX or precisely recognize the D3 epitope, mouse sera were tested by ELISA for binding to biotinylated D3 peptide immobilized on solid phase of microtiter plates. While the first boost immunization resulted in a 10-fold increase of anti-CgoX and anti-CgoX-D3 specific antibody concentrations, a second boost did not improve the antibody response. Compared to D3-CRM, the D3-BSA conjugate induced a more variable anti-CgoX and anti-CgoX-D3 antibody response including non-responders at initial immunization.

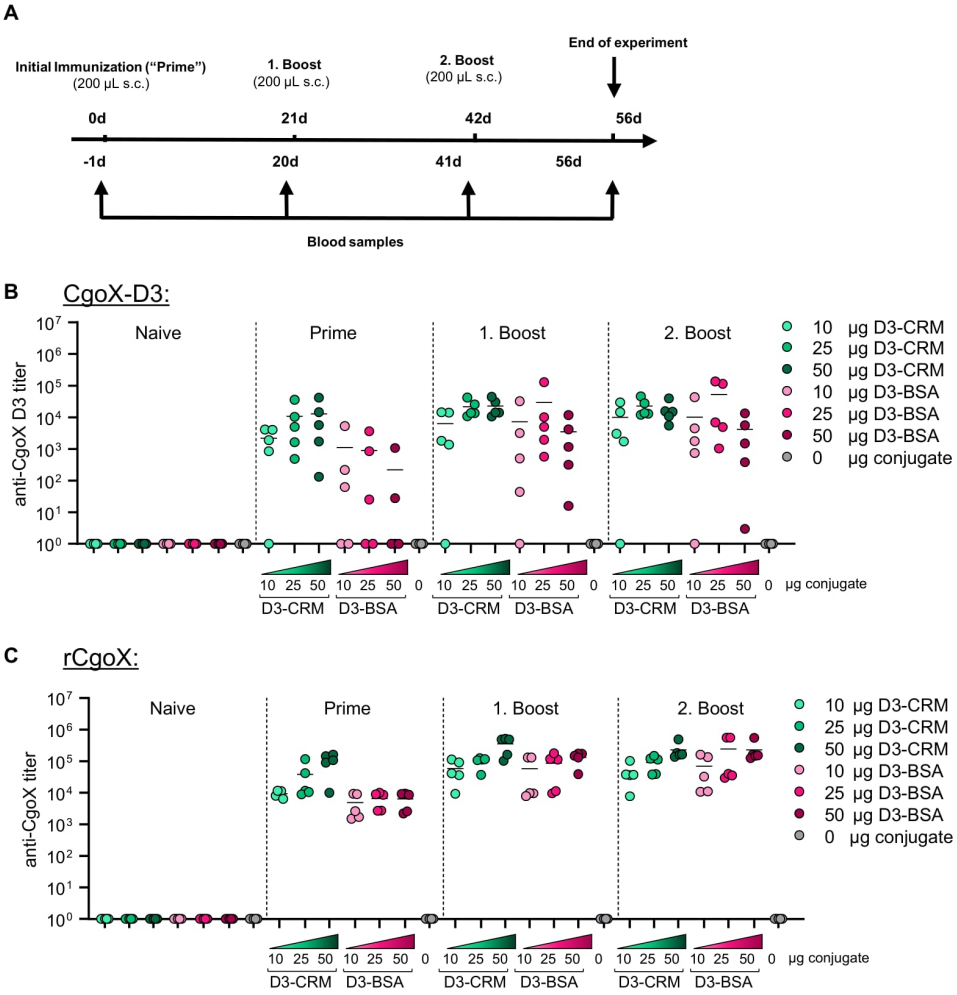


Figure 3. Dose-dependent antibody responses elicited by full-length CgoX protein or CgoX-D3 epitope in sera of immunized mice. (**A**) Female BALB/c mice ($n = 5$/group) were immunized subcutaneously on days 0, 21, and 42 with increasing doses of D3-CRM or D3-BSA (0, 10, 25, or 50 µg) formulated in PBS with 25% alhydrogel. Blood samples were collected one day prior to each immunization (days −1, 20, and 41) and two weeks after the final dose (day 56) via cheek punch from the vena facialis superficialis. (**B**,**C**) IgG antibodies against CgoX-D3 epitope (**B**) or the full-length CgoX protein (**C**) were measured by ELISA. Serum was analyzed using 4-fold serial dilutions starting at 1:250 (CgoX), or using 3-fold serial dilutions starting at 1:250 (CgoX-D3), and titers are expressed as the reciprocal of the highest dilution exceeding the cutoff (mean blank + 3 × SD). Data are shown as scatter plots with individual points for each animal and horizontal lines indicating group means.

3.3. Prime–Boost Combination Studies

The immune response to B-cell epitopes can be biased by the initial priming antigen. A full-length antigen used for initial immunization directs the immune response to dominant B-cell epitopes. Subsequent immunizations may be cued to boost the primary immune responses towards these dominant B-cell epitopes, while responses to subdominant epitopes are suppressed [23]. In humans, anti-CgoX specific antibodies are found at high frequency, whereas anti-CgoX-D3 specific antibodies are rarely detected (Figures 1 and 2). The question arose whether CgoX immunity in humans prevents the generation of antibodies specific for the CgoX-D3 epitope. We therefore tested whether D3-CRM can induce CgoX-D3 antibodies in mice previously primed with full-length CgoX. Prime–boost combinations of full-length CgoX and D3-CRM were compared for their ability to elicit anti-CgoX-D3 specific antibodies.

Two groups of mice were initially immunized with either D3-CRM or rCgoX. After 21 days, each group was split and boosted by either rCgoX or D3-CRM, revealing four

groups of prime–boost combinations (Figure 4B,C). At day 42, mice of each group received a second boost with D3-CRM. Sera of individual mice of each group were individually tested before and after any immunization for the presence of anti-D3-epitope and CgoX-specific antibodies (Figure 4B,C). Prior to vaccination, CgoX-specific antibodies were not detected in any of the sera, reflecting the immunological naivety of mice with respect to *S. aureus* antigens when kept at specific pathogen-free conditions.

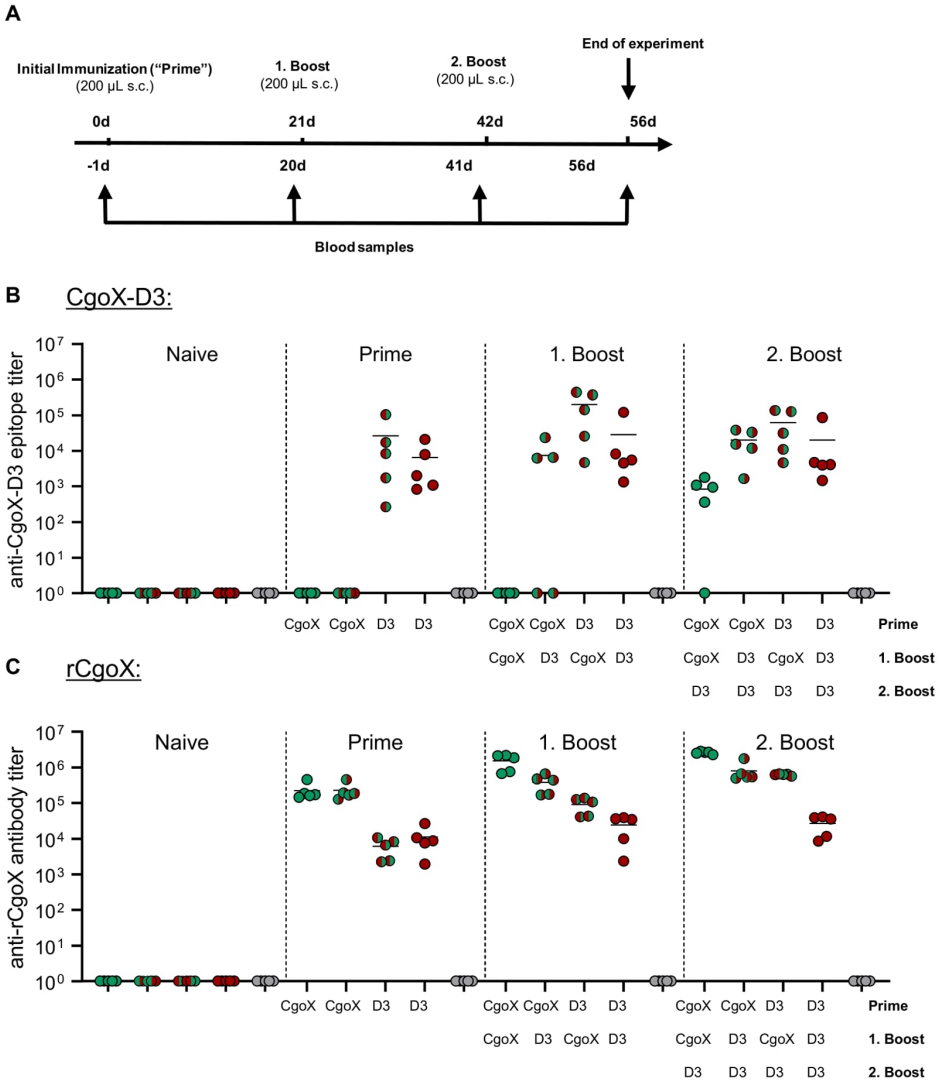


Figure 4. Antibody responses following prime–boost immunizations with full-length CgoX, CgoX-D3 epitope or combinations thereof. (**A**) Female BALB/c mice ($n = 5$/group) were immunized subcutaneously on days 0, 21, and 42 with full-length CgoX protein, CgoX-D3 epitope, or combinations thereof in a prime–boost regimen formulated in PBS with 25% alhydrogel. Blood samples were collected one day prior to each immunization (days −1, 20, and 41) and two weeks after the final dose (day 56) via cheek punch from the vena facialis superficialis. (**B**,**C**) IgG antibodies against the full-length CgoX protein (**B**) or CgoX-D3 epitope (**C**) were measured by ELISA. Serum was analyzed using 4-fold serial dilutions starting at 1:250 (CgoX), or using 3-fold serial dilutions starting at 1:250 (CgoX-D3), and titers are expressed as the reciprocal of the highest dilution exceeding the cutoff (mean blank + 3 × SD). Data are shown as scatter plots with individual points for each animal and horizontal lines indicating group means.

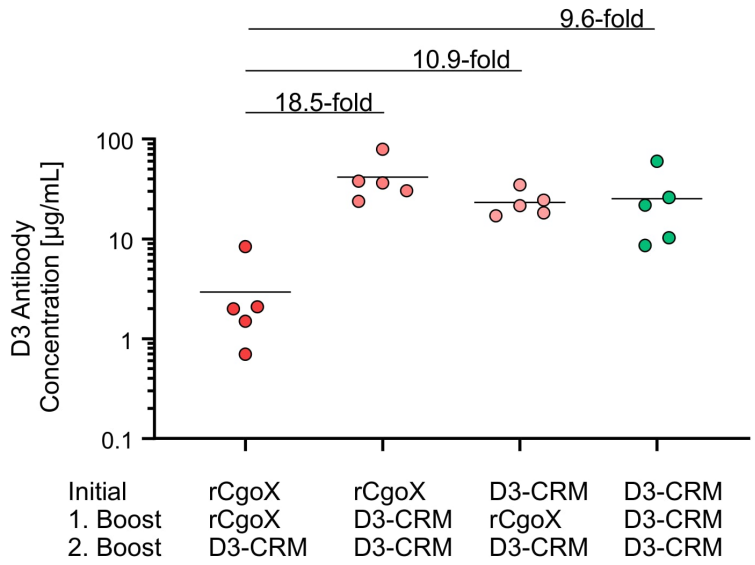
Initial immunization with rCgoX resulted in high titers of CgoX-specific antibodies, yet did not elicit anti-D3-epitope antibodies (Figure 4B,C), indicating the immunologically subdominant nature of the D3 epitope. In contrast, immunization with D3-CRM induced a D3

epitope antibody response (Figure 4B), which likely accounts for the observed recognition of full-length CgoX (Figure 4C).

The homologous boost of CgoX-primed mice with CgoX did not induce a detectable anti-D3-epitope response (Figure 4C), strongly underscoring the immunologic subdominance of the D3 epitope. Strikingly, boosting D3-CRM-primed mice with full-length CgoX produced high titers of D3-epitope-specific antibodies (Figure 4B), indicating a strong recall response to the subdominant D3 epitope. Notably, D3-CRM elicited D3-specific antibodies, although at lower titers, in mice primed and boosted with CgoX, suggesting that vaccination with a subdominant antigen breaks the interference of the CgoX-related immunological imprints.

This trend was quantitatively confirmed by a reference-standard-anchored ELISA by using a monoclonal anti-CgoX-D3 antibody. Priming with D3-CRM resulted in higher concentrations of D3-epitope-specific antibodies (Figure 5). When CgoX was used for priming and boost, the D3-specific antibody response to D3-CRM was significantly weaker compared to the other prime–boost combinations.

A



B

	CgoX / CgoX	CgoX / D3-CRM	D3-CRM / CgoX	D3-CRM / D3-CRM
Fold-difference	-	18.5	10.9	9.6
95% confidence interval	-	6.5 to 52.2	4.1 to 29.0	2.8 to 32.9
Adjusted p value	-	0.000576	0.000942	0.002823

Figure 5. Concentration of epitope-specific IgG in sera of immunized mice after the second boost. Sera of female BALB/c mice (n = 5/group) after the second boost (Figure 4A) were reanalyzed for epitope-specific IgG concentrations. Using an improved method with a linker and a standard curve generated from purified CgoX-D3-specific monoclonal antibody (0.1 µg/mL starting concentration, 3-fold serial dilutions); serum samples were diluted 1:100 and then 3-fold serially diluted. (**A**) CgoX-D3-specific concentrations were determined by interpolation from a standard curve generated using purified CgoX-D3-specific monoclonal antibody and blank-subtracted absorbance values, applying non-linear regression. Concentrations are expressed in µg/mL. Data are shown as scatter plots with individual points for each animal and horizontal lines indicating group means. Fold differences in antibody concentrations are shown relative to the group primed and boosted with recombinant CgoX protein (leftmost group). (**B**) Fold differences and 95% confidence intervals are shown relative to the

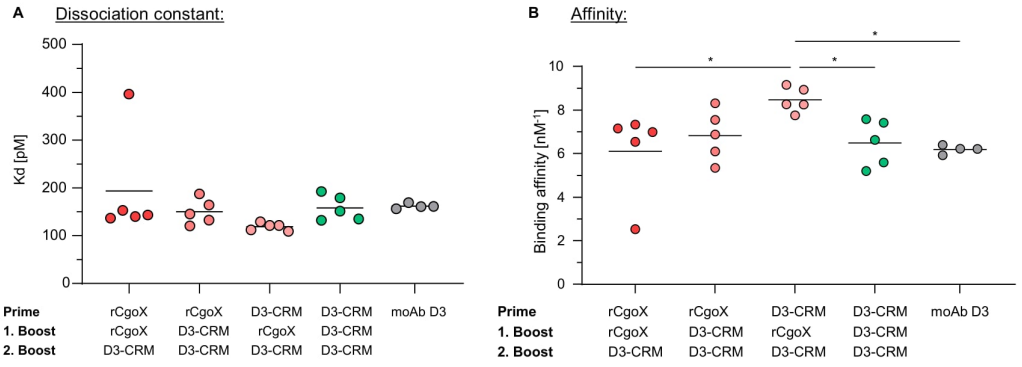
group primed and boosted with recombinant CgoX protein. Statistical analysis was performed using a lognormal *t*-test (unpaired *t*-test, assuming a lognormal distribution and equal geometric standard deviations). *p*-values were adjusted for multiple comparisons using the Holm–Šídák method ($\alpha = 0.05$).

Collectively, these data suggest that the D3 epitope is a subdominant *S. aureus* antigen, where even repeated immunization with full-length CgoX does not result in the generation of D3-specific antibodies. Targeted immunization with D3-CRM of naive or CgoX-primed mice is needed and sufficient to partially overcome interference from prior CgoX priming and to induce antibodies against the subdominant D3 epitope.

3.4. Binding Affinity of D3-Specific Antibodies

A monoclonal, anti-CgoX-D3-epitope specific antibody has been shown to be particularly protective in a mouse model of sepsis [22]. In general, the binding affinity is an important determinant of antibody potency. We therefore investigated the affinity of the anti-D3 antibodies induced by the individual prime–boost combinations. To this end, the same sera of the prime–boost experiment described in Figure 4 were re-analyzed by a D3-epitope-specific affinity ELISA using streptavidin-coated microtiter plates with an immobilized biotinylated CgoX-D3 peptide carrying an additional TTDS linker as the solid phase.

To enable direct comparison of antibody affinities, all serum samples were first normalized to an identical starting CgoX-D3-specific IgG concentration, as determined by the previous ELISA (Figure 5). Subsequent affinity ELISA analysis based on serial antibody dilutions revealed the lowest dissociation constant for the prime–boost combination D3-CRM/CgoX/D3-CRM (Figure 6A) corresponding to the statistically significant largest association constant or affinity (1/Kd) (Figure 6B). When anchored to the reference standard for the monoclonal anti-CgoX-D3 antibody (GMP-compliant anti-CgoX-D3 drug substance), the relative ELISA potency of the anti-CgoX-D3 specific antibodies induced by the D3-CRM/CgoX/D3-CRM prime–boost combination was 136.8% of the reference standard. These data suggest that a prime–boost strategy using D3-CRM is suitable for generating a high-affinity D3-specific antibody response.



C

Prime-Boost:	CgoX / CgoX	CgoX / D3-CRM	D3-CRM / CgoX	D3-CRM / D3-CRM	moAb D3
Kd [pM]	193.7	150.0	118.6	157.9	161.8
Affinity [nM^{-1}]	6.1	6.8	8.5	6.5	6.2
Potency	98.7 %	110.4 %	136.8 %	104.7 %	

Figure 6. Epitope-specific IgG affinity and binding parameters of CgoX-D3 antibodies following different prime–boost regimens. Sera of female BALB/c mice ($n = 5$/group) after the second boost (Figure 4A) were reanalyzed for binding parameters. Mouse sera were adjusted to a CgoX-D3-specific

antibody concentration of 0.1 µg/mL, as determined by the quantification ELISA in Figure 5. Epitope-specific binding parameters were measured using purified CgoX-D3-specific monoclonal antibody as well as serum samples adjusted to a starting concentration of 0.1 µg/mL and then 3-fold serially diluted. Binding parameters were calculated from blank-subtracted values using non-linear regression: (**A**) dissociation constant (Kd) and (**B**) affinity (1/Kd). Panel (**C**) summarizes the mean Kd and affinity for each group. Data are presented as scatter plots with individual points for each animal and horizontal lines indicating group means, with panel (**C**) showing group means. Statistical significance for differences in affinity (**B**) between groups was assessed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test, with *p*-values adjusted to control the false discovery rate (FDR) using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (Q = 0.05). Asterisks indicate discoveries following FDR correction ($q < Q$); all other pairwise comparisons did not meet the FDR threshold ($q \geq Q$).

4. Discussion

The present study addresses immunodominance and subdominance antibody responses in antimicrobial immunity, in which subdominance may represent a pathogen-driven immune evasion strategy [32]. There is an apparent disconnect between robust antibody titers recognizing *S. aureus* antigens in humans and the lack of protective immunity [9–11]. Recent work suggests that immune imprinting by immunodominant *S. aureus* antigens can attenuate recall responses to subdominant determinants [23], implying that subdominant regions may represent important targets for protective vaccination. Accumulating evidence, however, suggests that subdominant regions of *S. aureus* antigens potentially are key targets for protective immunization strategies [32]. Here, we show that the D3 epitope of the vaccine candidate CgoX exemplifies such a subdominant target that is rarely immunologically recognized at meaningful levels in individuals naturally exposed to *S. aureus*.

The majority of adult healthy blood donors harbor antibodies against the whole CgoX protein, but only a small fraction of humans exhibits D3-specific responses, and these are typically of low magnitude. Even in the context of invasive *S. aureus* infection, where antigen exposure is increased, D3-specific antibodies remain infrequent and low in concentration, suggesting a subdominant status of the D3 epitope, against which naturally acquired humoral immunity is limited. Independent evidence for the subdominance of the D3 epitope is provided by immunization studies with whole CgoX in mice. In contrast to humans, laboratory mice are not exposed to *S. aureus* and are immunologically naive. Mice primed and boosted with whole CgoX alone appear to direct the immune response away from D3-specific determinants. These findings support the immunological subdominance of the D3 epitope in both naturally exposed humans and experimentally immunized mice.

Subdominant antigens like the CgoX-D3 epitope have been shown to be promising targets for protective immunization in several major human pathogens including the hypervariable region (HVR) of the M protein of *Streptococcus pyogenes* [25,26], the amino-terminal domains of Rib and alpha proteins from *Streptococcus agalactiae* [27], and the HA stem of hemagglutinin (HA) of influenza virus [28]. Targeting of subdominant antigenic domains has also been proposed as a therapeutic strategy for *S. aureus* infections [23]. However, due to the unclear immunogenicity of these antigens in humans, clinical vaccine approaches have mostly targeted immunodominant antigens that already demonstrate robust but non-protective immunogenicity. At this point it is worth emphasizing that in general, antigenic subdominance refers to a quantitative difference, not a qualitative one. Thus, the subdominance of the D3 epitope may qualify CgoX as a target for protective antibodies, which has been demonstrated in mice [22].

A serious obstacle for using the subdominant D3 epitope for vaccination may be caused by immunological imprints induced by pre-existing immunity to whole CgoX. Indeed, non-protective immune imprints as a result of repeated and long-time exposure to immunodominant *S. aureus* antigens may interfere with subdominant but potentially protective determinants [12,23]. We here show that in mice, primed and boosted with CgoX, the D3-epitope vaccination can partially overcome the effects of prior CgoX priming. While the antibody response in CgoX-primed mice was quantitatively reduced, the D3-specific antibodies induced displayed a similar binding affinity to the cognate epitope, which is particularly relevant, as antibody affinity is a key determinant of functional activity. The attenuation of D3-specific antibody responses following CgoX priming is consistent with immunological imprinting. However, the experimental design does not address other mechanisms like epitope masking by pre-existing antibodies or competition among B-cell populations. Collectively, the data suggest that epitope-specific immunization can partially overcome negative immunological imprinting associated with CgoX priming, making the D3 epitope an attractive target for focused vaccination.

This study has some limitations. The immunogenicity of the D3-CRM conjugate in mice does not necessarily mean protective efficacy in humans. Previous studies demonstrated in vivo protective activity of D3-directed immunity using a D3-BSA conjugate [22]. The in vivo activity of humoral immunity elicited by D3-CRM197 conjugate has not been addressed. Carrier proteins and adjuvants influence the magnitude and quality of antibody responses, including IgG subclass distribution and associated Fc-mediated effector functions. In addition, the immune responses in mice cannot be directly translated into human immunity. Therefore, the robust D3-specific antibody responses elicited by D3-CRM197 immunization needs to be established in clinical trials.

Furthermore, the role of cellular immunity, particularly Th1 and Th17 responses, was not assessed but is increasingly recognized as critical in defense against *S. aureus*. Increasing evidence indicates that protective immunity against *S. aureus* depends not only on functional antibodies but also on antigen-specific $CD4^+$ T-cell responses, particularly Th1 and Th17 cells, which promote neutrophil recruitment, macrophage activation, and bacterial clearance [33]. Finally, although the D3 epitope is highly conserved, diverse clinical *S. aureus* isolates and individual patients' immune status may affect the immunological response to D3-CRM vaccination.

Although animals were not randomly allocated to the experimental groups and not analyzed under blinded conditions, the primary outcome measures were based on serological analyses, rather than subjective assessments of the animals. The relatively small group size ($n = 5$ per group) limits the statistical power and generalizability of the findings.

In conclusion, our findings identify the CgoX-D3 epitope as a promising target for further investigation in epitope-based vaccine development against *S. aureus*. The D3 epitope is rarely recognized at substantial levels in naturally exposed humans, yet targeted immunization with D3-CRM induces high-affinity epitope-specific antibodies and can partially overcome interference from prior CgoX priming. These results provide the immunogenicity feasibility of D3-focused immunization and support a rational path forward aiming at redirecting immunity away from immunodominant, non-protective determinants and toward conserved protective epitopes.

Author Contributions: Conceptualization, M.K., L.M.M. and S.M.; methodology, S.M., L.M.M. and M.K.; validation, L.M.M. and M.K.; formal analysis, L.M.M. and A.G.; investigation L.M.M., S.M., A.G., D.G. and K.W.; resources, B.G., O.A.C. and J.S.; data curation, L.M.M. and A.K.; writing—original draft preparation, L.M.M. and M.K.; writing—review and editing, L.M.M. and M.K.; visualization, L.M.M.; supervision, M.K.; project administration, L.M.M.; funding acquisition, M.K. and A.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of the University of Cologne and the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection, Veterinary Section, Recklinghausen, Germany (approval number AZ 81-02.04.2020.A163, approved on 10.08.2020), in accordance with the Animal Protection Law of Germany. The studies involving human samples were conducted in accordance with the Declaration of Helsinki and the guidelines of Good Clinical Practice (CPMP/ICH/135/95), and were approved by the Ethics Committee of the Medical Faculty of the University of Cologne, Germany (registration number 21-1561, approved on 02.03.2022, for the healthy blood donor sera; registration number 08-160, approved on 22.05.2019, for the clinical plasma cohort).

Informed Consent Statement: Written informed consent was obtained from all blood donors under the established procedures of the Institute of Transfusion Medicine, University Hospital Cologne, prior to blood donation. [For the clinical cohort: either "Written informed consent was obtained from all patients included in the clinical cohort" or "The requirement for informed consent for the use of archived plasma samples from the clinical cohort was waived by the Ethics Committee of the Medical Faculty of the University of Cologne (registration number 08-160)."] This study involved analyses of previously collected human serum and plasma samples and did not involve prospective intervention in human participants.

Data Availability Statement: All raw data of this study are archived on the institutional server and will be made available by the authors on reasonable request.

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Conflicts of Interest: M.K. is patentholder of EP 1987836 Protective staphylococcus aureus vaccine based on cell wall-associated proteins. M.K. and A.K. are patentholders of PCT/US 11,801,292 B2. All other authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CgoX	Coproporphyrinogen III oxidase
CRM197	Cross-reactive material 197
D3-CRM	CgoX-D3–CRM197 conjugate
FnBPA	Fibronectin-binding protein A
GapC	Glyceraldehyde-3-phosphate dehydrogenase C
HVR	Hypervariable region
IsdB	Iron-regulated surface determinant B
Kd	Dissociation constant
mAb	Monoclonal antibody
MntC	Manganese transport protein C
rFSAV	Recombinant five-antigen *Staphylococcus aureus* vaccine
Ttds	4,7,10-trioxa-1,13-tridecanediamino succinic acid linker
HA	Hemagglutinin

Appendix A

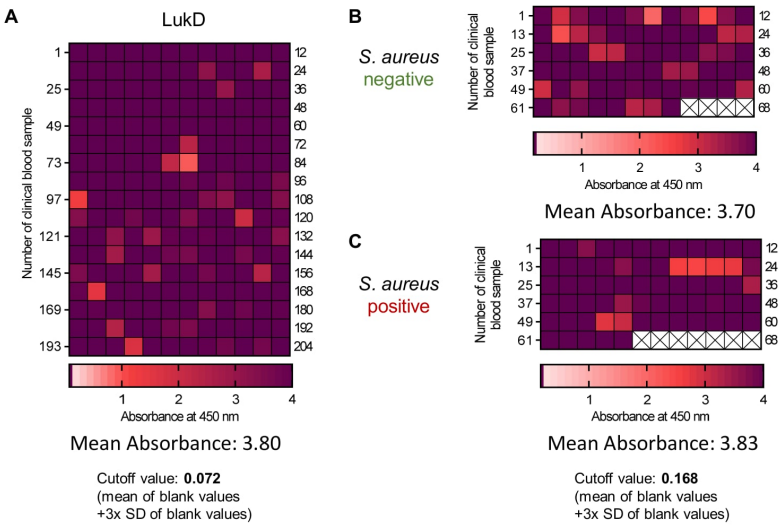


Figure A1. Seroprevalence of anti-LukD specific antibodies. The anti-LukD antibody response was analyzed by indirect ELISA with recombinant $LukD_{His6}$ on solid phase. Visualized is the absorbance at 450 nm. Abundance of anti-LukD response among (**A**) serum samples from 204 healthy donors, (**B**) plasma samples from 68 COVID-19 study participants not infected with *S. aureus* and (**C**) 65 plasma samples from 65 COVID-19 study participants with invasive *S. aureus* infection is shown.

Appendix B

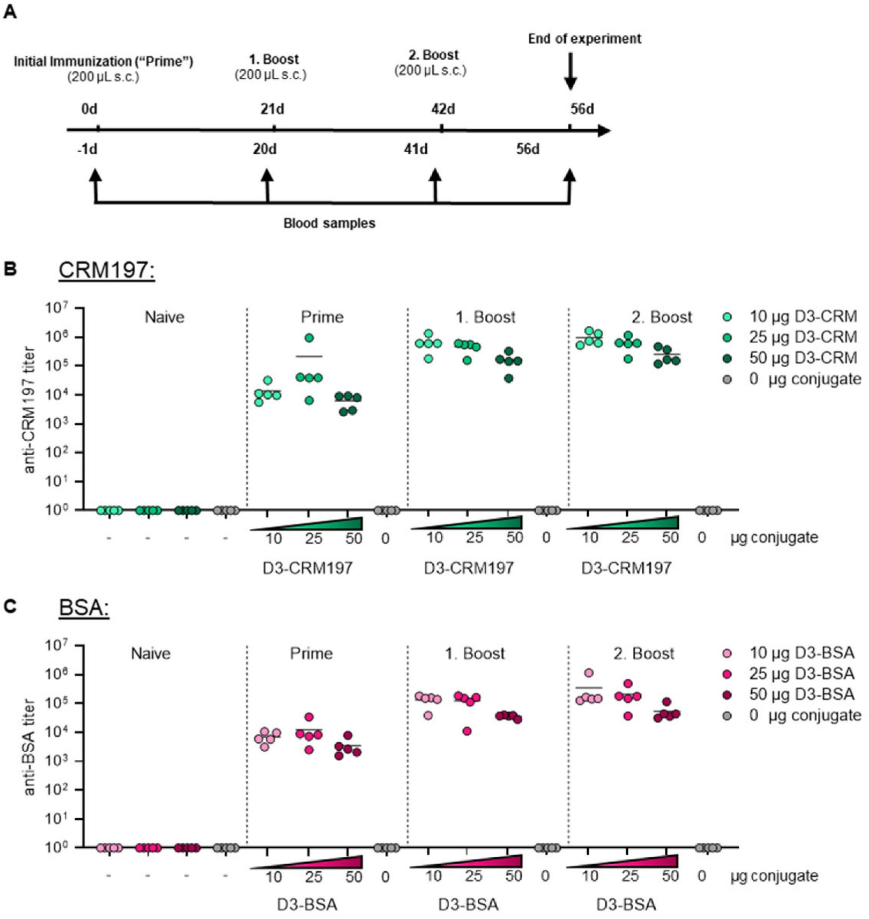


Figure A2. Dose-dependent antibody responses to carrier molecules CRM197 and BSA in sera of immunized mice to assess immunogenicity. (**A**) Female BALB/c mice ($n = 5$/group) were immunized

subcutaneously on days 0, 21, and 42 with increasing doses of D3-CRM or D3-BSA (0, 10, 25, or 50 μg) formulated in PBS with 25% alhydrogel. Blood samples were collected one day prior to each immunization (days −1, 20, and 41) and two weeks after the final dose (day 56) via cheek punch from the vena facialis superficialis. (**B**,**C**) IgG antibodies against the carrier CRM197 (**B**) or the carrier BSA (**C**) were measured by ELISA. Serum was analyzed using 4-fold serial dilutions starting at 1:250 (CgoX), and titers are expressed as the reciprocal of the highest dilution exceeding the cutoff (mean blank + 3 × SD). Data are shown as scatter plots with individual points for each animal and horizontal lines indicating group means.

References

1. Tong, S.Y.C.; Davis, J.S.; Eichenberger, E.; Holland, T.L.; Fowler, V.G. *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. *Clin. Microbiol. Rev.* **2015**, *28*, 603–661. [CrossRef] [PubMed]
2. Antimicrobial Resistance Collaborators. Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *Lancet* **2022**, *399*, 629–655. [CrossRef] [PubMed]
3. GBD 2021 Antimicrobial Resistance Collaborators. Global Burden of Bacterial Antimicrobial Resistance 1990–2021: A Systematic Analysis with Forecasts to 2050. *Lancet* **2024**, *404*, 1199–1226. [CrossRef] [PubMed]
4. Fowler, V.G.; Proctor, R.A. Where Does a *Staphylococcus aureus* Vaccine Stand? *Clin. Microbiol. Infect.* **2014**, *20*, 66–75. [CrossRef] [PubMed]
5. Miller, L.S.; Fowler, V.G.; Shukla, S.K.; Rose, W.E.; Proctor, R.A. Development of a Vaccine against *Staphylococcus aureus* Invasive Infections: Evidence Based on Human Immunity, Genetics and Bacterial Evasion Mechanisms. *FEMS Microbiol. Rev.* **2020**, *44*, 123–153. [CrossRef] [PubMed]
6. Clegg, J.; Soldaini, E.; McLoughlin, R.M.; Rittenhouse, S.; Bagnoli, F.; Phogat, S. *Staphylococcus aureus* Vaccine Research and Development: The Past, Present and Future, Including Novel Therapeutic Strategies. *Front. Immunol.* **2021**, *12*, 705360. [CrossRef] [PubMed]
7. Redi, D.; Raffaelli, C.S.; Rossetti, B.; De Luca, A.; Montagnani, F. *Staphylococcus aureus* Vaccine Preclinical and Clinical Development: Current State of the Art. *New Microbiol.* **2018**, *41*, 208–213. [PubMed]
8. Proctor, R.A. Immunity to *Staphylococcus aureus*: Implications for Vaccine Development. *Microbiol. Spectr.* **2019**, *7*, 4. [CrossRef] [PubMed]
9. Fritz, S.A.; Tiemann, K.M.; Hogan, P.G.; Epplin, E.K.; Rodriguez, M.; Al-Zubeidi, D.N.; Bubeck Wardenburg, J.; Hunstad, D.A. A Serologic Correlate of Protective Immunity against Community-Onset *Staphylococcus aureus* Infection. *Clin. Infect. Dis.* **2013**, *56*, 1554–1561. [CrossRef] [PubMed]
10. Meyer, T.C.; Michalik, S.; Holtfreter, S.; Weiss, S.; Friedrich, N.; Völzke, H.; Kocher, T.; Kohler, C.; Schmidt, F.; Bröker, B.M.; et al. A Comprehensive View on the Human Antibody Repertoire Against *Staphylococcus aureus* Antigens in the General Population. *Front. Immunol.* **2021**, *12*, 651619. [CrossRef] [PubMed]
11. Radke, E.E.; Brown, S.M.; Pelzek, A.J.; Fulmer, Y.; Hernandez, D.N.; Torres, V.J.; Thomsen, I.P.; Chiang, W.K.; Miller, A.O.; Shopsin, B.; et al. Hierarchy of Human IgG Recognition within the *Staphylococcus aureus* Immunome. *Sci. Rep.* **2018**, *8*, 13296. [CrossRef] [PubMed]
12. Tsai, C.-M.; Caldera, J.R.; Hajam, I.A.; Chiang, A.W.T.; Tsai, C.-H.; Li, H.; Díez, M.L.; Gonzalez, C.; Trieu, D.; Martins, G.A.; et al. Non-Protective Immune Imprint Underlies Failure of *Staphylococcus aureus* IsdB Vaccine. *Cell Host Microbe* **2022**, *30*, 1163–1172.e6. [CrossRef] [PubMed]
13. Fowler, V.G.; Allen, K.B.; Moreira, E.D.; Moustafa, M.; Isgro, F.; Boucher, H.W.; Corey, G.R.; Carmeli, Y.; Betts, R.; Hartzel, J.S.; et al. Effect of an Investigational Vaccine for Preventing *Staphylococcus aureus* Infections after Cardiothoracic Surgery: A Randomized Trial. *JAMA* **2013**, *309*, 1368–1378. [CrossRef] [PubMed]
14. Katzelnick, L.C.; Gresh, L.; Halloran, M.E.; Mercado, J.C.; Kuan, G.; Gordon, A.; Balmaseda, A.; Harris, E. Antibody-Dependent Enhancement of Severe Dengue Disease in Humans. *Science* **2017**, *358*, 929–932. [CrossRef] [PubMed]
15. Rappuoli, R.; Bottomley, M.J.; D'Oro, U.; Finco, O.; De Gregorio, E. Reverse Vaccinology 2.0: Human Immunology Instructs Vaccine Antigen Design. *J. Exp. Med.* **2016**, *213*, 469–481. [CrossRef] [PubMed]
16. Li, J.; Ju, Y.; Jiang, M.; Li, S.; Yang, X.-Y. Epitope-Based Vaccines: The Next Generation of Promising Vaccines Against Bacterial Infection. *Vaccines* **2025**, *13*, 248. [CrossRef] [PubMed]
17. Zeng, H.; Zhang, J.; Song, X.; Zeng, J.; Yuan, Y.; Chen, Z.; Xu, L.; Gou, Q.; Yang, F.; Zeng, N.; et al. An Immunodominant Epitope-Specific Monoclonal Antibody Cocktail Improves Survival in a Mouse Model of *Staphylococcus aureus* Bacteremia. *J. Infect. Dis.* **2021**, *223*, 1743–1752. [CrossRef] [PubMed]
18. Yang, H.-J.; Zhang, J.-Y.; Wei, C.; Yang, L.-Y.; Zuo, Q.-F.; Zhuang, Y.; Feng, Y.-J.; Srinivas, S.; Zeng, H.; Zou, Q.-M. Immunisation With Immunodominant Linear B Cell Epitopes Vaccine of Manganese Transport Protein C Confers Protection against *Staphylococcus aureus* Infection. *PLoS ONE* **2016**, *11*, e0149638. [CrossRef] [PubMed]

19. Chen, Z.; Gou, Q.; Xiong, Q.; Duan, L.; Yuan, Y.; Zhu, J.; Zou, J.; Chen, L.; Jing, H.; Zhang, X.; et al. Immunodominance of Epitopes and Protective Efficacy of HI Antigen Are Differentially Altered Using Different Adjuvants in a Mouse Model of *Staphylococcus aureus* Bacteremia. *Front. Immunol.* **2021**, *12*, 684823. [CrossRef] [PubMed]
20. Ma, J.; Wei, Y.; Zhang, L.; Wang, X.; Yao, D.; Liu, D.; Liu, W.; Yu, S.; Yu, Y.; Wu, Z.; et al. Identification of a Novel Linear B-Cell Epitope as a Vaccine Candidate in the N2N3 Subdomain of *Staphylococcus aureus* Fibronectin-Binding Protein A. *J. Med. Microbiol.* **2018**, *67*, 423–431. [CrossRef] [PubMed]
21. Soltan, M.A.; Magdy, D.; Solyman, S.M.; Hanora, A. Design of *Staphylococcus aureus* New Vaccine Candidates with B and T Cell Epitope Mapping, Reverse Vaccinology, and Immunoinformatics. *OMICS* **2020**, *24*, 195–204. [CrossRef] [PubMed]
22. Klimka, A.; Mertins, S.; Nicolai, A.K.; Rummler, L.M.; Higgins, P.G.; Günther, S.D.; Tosetti, B.; Krut, O.; Krönke, M. Epitope-Specific Immunity against *Staphylococcus aureus* Coproporphyrinogen III Oxidase. *npj Vaccines* **2021**, *6*, 11. [CrossRef] [PubMed]
23. Caldera, J.R.; Tsai, C.-M.; Trieu, D.; Gonzalez, C.; Hajam, I.A.; Du, X.; Lin, B.; Liu, G.Y. The Characteristics of Pre-Existing Humoral Imprint Determine Efficacy of *S. aureus* Vaccines and Support Alternative Vaccine Approaches. *Cell Rep. Med.* **2024**, *5*, 101360. [CrossRef] [PubMed]
24. Teymournejad, O.; Li, Z.; Beesetty, P.; Yang, C.; Montgomery, C.P. Toxin Expression during *Staphylococcus aureus* Infection Imprints Host Immunity to Inhibit Vaccine Efficacy. *npj Vaccines* **2023**, *8*, 3. [CrossRef] [PubMed]
25. Lannergård, J.; Gustafsson, M.C.U.; Waldemarsson, J.; Norrby-Teglund, A.; Stålhammar-Carlemalm, M.; Lindahl, G. The Hypervariable Region of *Streptococcus pyogenes* M Protein Escapes Antibody Attack by Antigenic Variation and Weak Immunogenicity. *Cell Host Microbe* **2011**, *10*, 147–157. [CrossRef] [PubMed]
26. Beachey, E.H.; Seyer, J.M.; Dale, J.B. Protective Immunogenicity and T Lymphocyte Specificity of a Trivalent Hybrid Peptide Containing NH2-Terminal Sequences of Types 5, 6, and 24 M Proteins Synthesized in Tandem. *J. Exp. Med.* **1987**, *166*, 647–656. [CrossRef] [PubMed]
27. Stålhammar-Carlemalm, M.; Waldemarsson, J.; Johnsson, E.; Areschoug, T.; Lindahl, G. Nonimmunodominant Regions Are Effective as Building Blocks in a Streptococcal Fusion Protein Vaccine. *Cell Host Microbe* **2007**, *2*, 427–434. [CrossRef] [PubMed]
28. Wei, C.-J.; Crank, M.C.; Shiver, J.; Graham, B.S.; Mascola, J.R.; Nabel, G.J. Next-Generation Influenza Vaccines: Opportunities and Challenges. *Nat. Rev. Drug Discov.* **2020**, *19*, 239–252. Correction in: *Nat. Rev. Drug Discov.* **2020**, *19*, 427. https://doi.org/10.1038/s41573-020-0066-8. [CrossRef] [PubMed]
29. Glowalla, E.; Tosetti, B.; Krönke, M.; Krut, O. Proteomics-Based Identification of Anchorless Cell Wall Proteins as Vaccine Candidates against *Staphylococcus aureus*. *Infect. Immun.* **2009**, *77*, 2719–2729. [CrossRef] [PubMed]
30. Pichichero, M.E. Protein Carriers of Conjugate Vaccines: Characteristics, Development, and Clinical Trials. *Hum. Vaccin. Immunother.* **2013**, *9*, 2505–2523. [CrossRef] [PubMed]
31. Zhao, F.; Zai, X.; Zhang, Z.; Xu, J.; Chen, W. Challenges and Developments in Universal Vaccine Design against SARS-CoV-2 Variants. *npj Vaccines* **2022**, *7*, 167. [CrossRef] [PubMed]
32. Lindahl, G. Subdominance in Antibody Responses: Implications for Vaccine Development. *Microbiol. Mol. Biol. Rev.* **2020**, *85*, e00078-20. [CrossRef] [PubMed]
33. Armentrout, E.I.; Liu, G.Y.; Martins, G.A. T Cell Immunity and the Quest for Protective Vaccines against *Staphylococcus aureus* Infection. *Microorganisms* **2020**, *8*, 1936. [CrossRef] [PubMed]

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