

Antibody responses in furunculosis patients vaccinated with autologous formalin-killed *Staphylococcus aureus*

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Abstract Autologous vaccines (short: autovaccines) have been used since the beginning of the 20th century to treat chronic staphylococcal infections, but their mechanisms of action are still obscure. This prospective pilot study involved four patients with furunculosis who were vaccinated with autologous formalin-killed *Staphylococcus aureus* cells. Vaccines were individually prepared from the infecting *S. aureus* strain and repeatedly injected subcutaneously in

increasing doses over several months. We characterized the virulence gene repertoire and *spa* genotype of the infecting and colonising *S. aureus* strains. Serum antibody responses to secreted and surface-bound bacterial antigens were determined by two-dimensional immunoblotting and flow-cytometry based assays (Luminex®). All patients reported clinical improvement. Molecular characterization showed that all strains isolated from one patient over time belonged to the same *S. aureus* clone. Already before treatment, there was robust antibody binding to a broad range of staphylococcal antigens. Autovaccination moderately boosted the IgG response to extracellular antigens in two patients, while the antibody response of the other two patients was not affected. Similarly, vaccination moderately enhanced the antibody response against some staphylococcal surface proteins, e.g. ClfA, ClfB, SdrD and SdrE. In summary, autovaccination only slightly boosted the pre-existing serum antibody response, predominantly to bacterial surface antigens.

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Introduction

Besides being a common colonizer of human skin and mucosa, *Staphylococcus aureus* also acts as a major human pathogen. The species can cause a broad range of infections, most frequently skin and soft tissue infections, such as wound infections, furuncles, carbuncles and abscesses, but also life-threatening systemic infections, such as pneumonia and sepsis [1–3]. Furunculosis is a common staphylococcal skin disease characterised by painful, deep infections of the hair follicle. Even mild lesions are painful and unsightly and often leave a scar after they heal [4]. Antibiotic treatment is frequently not effective, and many furunculosis patients suffer from recurrent episodes or develop chronic symptoms [4].

The alarming global spread of antibiotic resistant strains has spurred efforts to develop active and passive anti-staphylococcal vaccines [5, 6]. However, vaccine development is a challenging task, because both the species *S. aureus* and the host response that it induces are highly variable. Two *S. aureus* strains can differ drastically in their virulence gene content [7]. The variable genome consists of mobile genetic elements such as pathogenicity islands and phages that encode numerous virulence factors, including toxins, exoenzymes and immune modulators [7–10]. In concert with conserved virulence factors, these variable bacterial compounds could determine differential pathogenesis [11]. We observed a strong and strain-specific antibody response against these variable antigens in *S. aureus* carriers and during the natural course of *S. aureus* bacteremia [12].

Active vaccination can be based on mono- or multivalent subunit vaccines or on whole cell vaccines, which include autologous vaccines (short: autovaccines) [6]. Autovaccines are individually prepared from the autologous infecting bacterial strain [13, 14]. Following subculture, the bacteria and their secreted proteins are usually inactivated by fixation, heat or cell lysis, and then repeatedly applied orally or subcutaneously [13, 15]. In contrast to subunit vaccines, autovaccines contain poorly characterized varied cocktails of surface proteins and secreted virulence factors produced by the infecting strain.

Before the antibiotic era, chronic staphylococcal infections such as chronic furunculosis and osteomyelitis were frequently treated by therapeutic vaccination with autologous formalin-killed *S. aureus* cells [14, 16–18]. Today, autovaccination is still regularly performed in some Eastern European countries, including Poland and the Czech Republic [13, 19]. It is offered as a therapeutic alternative to patients with chronic *S. aureus* infections that are refractory to standard therapy. Moreover, bacterial whole cell vaccines are commonly used in veterinary medicine to treat chronic infectious diseases [20–22]. The major argument against the use of autovaccines in human medicine is that safety and efficacy have not been determined in controlled clinical trials. Moreover, the mode of action is largely unknown.

In this prospective pilot study we analyzed if autovaccination influences the serum antibody response to a broad spectrum of secreted and surface-bound *S. aureus* antigens.

Materials and methods

Autovaccination patients

The Department of Medical Microbiology and Immunology at the Pomeranian Medical University, Szczecin, Poland has

long-standing experience with autovaccination for therapy of chronic *S. aureus* furunculosis and osteomyelitis [13]. This prospective pilot study included four patients (3 female and 1 male) from the Szczecin area. They suffered from chronic or recurrent furunculosis and asked for autovaccination, one of the treatment options in Poland. In all four patients previous antibiotic treatment and surgical intervention had been unsuccessful. Patient data and anatomic location of the furuncles are shown in Table 1. The patients showed no signs of immune suppression. All four patients provided their written informed consent and the study was approved by the Ethics Board of the University of Szczecin.

S. aureus strains and sera

The *S. aureus* vaccine strains were isolated from infected lesions (pus) during episodes of furunculosis. Moreover, nasal swabs were taken repeatedly to determine the nasal colonization status. *S. aureus* was identified on the basis of generally accepted criteria: the presence of clumping factor (slide test) and extracellular coagulase (tube test), and biochemical identification (ID 32 Staph, BioMerieux). The infecting and colonizing *S. aureus* isolates were stored as glycerol stocks.

Antimicrobial susceptibility of isolates was estimated by the disc diffusion method according to current recommendations by the NCCLS/CLSI. Resistance to oxacillin/cefoxitin was confirmed by detection of PBP 2' (Slidex MRSA detection, BioMerieux) and *mecA* PCR (see below).

Sera were obtained directly before and during autovaccination treatment and stored at -80°C . We obtained four consecutive serum samples from patient 6466 (day 0, 86, 273 and 332), three samples from patients 7293 (day 0, 42, and 187) and 9105 (day 0, 85, and 166), and two samples from patient 7510 (day 0, and 63). Additionally, control sera from 11 healthy lab workers from the Department of Microbiology and Immunology, Pomeranian Medical University, Szczecin (age 37.6 ± 10.7 years; 36% male) were analyzed.

Preparation of autologous vaccines

Autovaccines were individually prepared from the causative *S. aureus* strain, which was isolated from furunculosis lesions. Autologous strains were cultivated for a maximum of 24 h on oblique test tubes containing PPLO agar (Becton Dickinson) supplemented with 10% (w/v) glucose and 0.1% (v/v) Tween 80. Bacteria were resuspended in 0.9% NaCl solution and fixed with 0.4% formalin solution for 48 h. The bacterial suspension was adjusted to 5×10^8 bacteria/ml (suspension I), 1×10^9 bacteria/ml (suspension II), and 2.5×10^9 bacteria/ml (suspension III) based on

Table 1 Clinical outcome of autovaccination

Patient data		Patient 6466	Patient 7293	Patient 7510	Patient 9105
Age (y)		23	35	50	41
Gender		Female	Female	Male	Female
Risk factors ^a		Atopic dermatitis	–	–	Atopic dermatitis
Onset of furuncles		02/06	07/06	2002	07/06
AV treatment		08/07–12/08	10/06–08/07	10/06–10/07	12/06–10/07
Location of furuncles		Face, neck, axilla	Axilla, abdomen, back	Face, neck	Axilla, buttocks
Mean number of episodes per month	Before AV	2	4	2	3.5
	During AV	0	1	2	0.2
	After AV	1	0	0.6	0
Chronicity	Before AV	Intermittently	Permanently	Permanently	Permanently
	During AV	Symptom free	Intermittently	Intermittently	Intermittently
	After AV	Intermittently	Symptom free	Intermittently	Symptom free
Single/multiple sites	Before AV	Multiple	Multiple	Multiple	Multiple
	During AV	–	Multiple(1 episode)	Single	Single
	After AV	Single	–	Single	–
Severity score ^b	Before AV	2	2	2	3
	During AV	0	1	1	1
	After AV	1	0	1	0
Clinical improvement ^c		Strong	Strong	Moderate	Strong

AV autovaccination

^aRisk factors: atopic dermatitis, chronic wounds, gout, diabetes mellitus, COPD, cancer, chronic liver disease, cardiovascular disease, rheumatoid diseases, HIV, chronic gut diseases (Morbus Crohn, Ulcerative Colitis), psoriasis, regular dialysis, immune suppressive drugs

^bDisease severity was assessed based on a severity score. 0 – No furuncles/abscesses; 1 – Few small furuncles, self-healing in less than two weeks, no medical intervention required; 2 – Furuncles of moderate severity, medical intervention required; 3 – Severe furuncles/carbuncles with strong pain and large fluid collections, medical intervention required

^cClinical improvement (classified as none, moderate or strong) based on the patients personal rating.

McFarland quantification. Afterwards, 0.1 mg/ml thiomersal was added to these suspensions as preservative. Finally, sterility of autovaccine preparation was tested by cultivation in thioglycollate medium (Becton Dickinson) for 7 days.

Administration of autovaccines

Autovaccines were prepared by the Department of Microbiology and Immunology, Pomeranian Medical University in Szczecin, and administered by the patients' general practitioners. Before the beginning of treatment, a skin test was performed to exclude hypersensitivity of the patient to any of the vaccine components. Intradermally, 0.5 ml of a 1:10 dilution of suspension I in Aqua ad injectionem was injected. Skin reaction was checked after 15 min and 24 hours. Autovaccines were then applied subcutaneously in 3–5 day intervals over 3 months. With each injection the dose was increased based on the following application scheme. Suspension I (5×10^8 bacteria/ml) was applied in volumes of 0.1, 0.2, 0.3, 0.4 and 0.5 ml, followed by

suspension II (1×10^9 bacteria/ml; 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml) and suspension III (2.5×10^9 bacteria/ml; 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml). This immunization procedure was followed by several recall injections (suspension III, 1 ml) every 1–3 weeks for up to 5–12 months. After each injection the patients were closely monitored for 30 minutes (swelling, pain or rash on injection side, blood pressure, breathing). After every vaccination, presumptive systemic symptoms (fever, headache, general malaise, sore throat, etc.) were also analyzed by a physician to exclude any side effects associated with autovaccination. Moreover, laboratory tests were performed before autovaccination and after completion of the basic application scheme (blood count, CRP; urine: proteinuria).

Clinical evaluation

The impact of autovaccination on the severity and frequency of furunculosis was retrospectively analyzed by reviewing the clinical records. Additionally, a telephone interview using a questionnaire was performed by

a physician, which included questions about the chronicity, duration, location and severity of furuncles, as well as a patient's rating of the clinical improvement after autovaccination.

Detection of *S. aureus* virulence factors by PCR

PCR was used to screen for a total of 26 virulence genes. Single and multiplex-PCR were applied for the detection of gyrase (*gyr*), methicillin resistance (*mecA*), Panton-Valentine-leukocidin (*pvl*), staphylococcal enterotoxins (*sea-seu*), toxic shock syndrome toxin 1 (*tst*), exfoliative toxins (*eta*, *etb*, *etd*), and *agr* groups 1–4 as previously reported [8].

spa genotyping

PCR for amplification of the *S. aureus* protein A (*spa*) repeat region was performed according to the published protocol [23, 24]. PCR products were sequenced by a commercial supplier using both amplification primers (Agowa, Berlin, Germany). The forward and reverse sequence chromatograms were analyzed with the Ridom StaphType software (Ridom GmbH, Würzburg, Germany). *Spa* types were clustered into different clonal clusters using the Based Upon Repeat Pattern (BURP) algorithm. Since *spa* typing and multilocus-sequence typing (MLST) are highly concordant, *spa* typing data could be mapped on MLST types by using the SpaServer database (www.spaserver.ridom.de).

2D-Immunoblots

Two-dimensional polyacrylamide gel electrophoresis (2-DE) with mini 2D gels and 2D immunoblots (2D-IBs) were performed as described [25]. Briefly, for antigen preparation, bacteria were inoculated in tryptic soy broth (TSB) to an optical density at 540 nm of 0.05 and cultivated in 100 ml cultures at 37°C and 180 rpm. Cultures were harvested 3.5 h after the bacterial culture entered the stationary phase, extracellular proteins were collected, and protein concentration was determined as previously described [11, 25].

Afterwards, isoelectric focusing was performed with 7 cm Immobiline Dry Strips of pI ranges 4–7 and 6–11 (GE Healthcare, Munich, Germany). Following separation according to molecular mass in the second dimension, the resolved staphylococcal proteins were blotted onto a PVDF membrane (Immobilon-P, Millipore) and incubated with the corresponding human sera at 1:10,000 dilution. IgG binding was detected by peroxidase-conjugated goat anti-human IgG and visualized with an ECL substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce). Consecutive serum samples from one patient were always

analyzed simultaneously; at least three technical replicates were performed for each patient.

Analysis of the 2D-IB images was performed with the Delta-2D software package version 4.0 (Decodon GmbH, Greifswald, Germany) as described [11, 25]. A fused image of all IBs from one patient was obtained in a two-step procedure. First, all IB images from one time course experiment were matched with the most complex IB and a fusion image was obtained using the union fuse option. Second, the fusion images from the three technical replicates were matched and fused. Spots on the fusion image were automatically detected and manually validated by comparing the original blot images with the fusion image. Subsequently, the spot map and the corresponding labels from the fusion image were transferred to all blot images from one patient. For spot detection on IBs the signal intensity threshold was set to 0.2 arbitrary units (AU).

Flamingo[®] protein staining

To correlate IB binding with protein abundance, representative IB images were matched with Flamingo[®]-stained 2D gels. Gels were stained with the Flamingo[®] Fluorescent Gel Stain (BioRad, Munich, Germany) according to the manufacturer's instructions.

Bead-based flow cytometry (Luminex[®])

Serum IgG, IgA and IgM directed against a panel of 40 recombinant staphylococcal antigens was quantified using the bead based flow cytometry technique (xMap[®], Luminex Corporation, Austin, Texas, USA). Antigens comprised surface proteins, such as clumping factor A and B (ClfA, ClfB), and serine-aspartate dipeptide repeat proteins (SdrD and SdrE), and secreted proteins, including toxins (superantigens, leukotoxins) and immune modulatory proteins (CHIPS, SCIN, SSL proteins).

Methods were as described previously [26, 27]. Tests were performed in independent duplicates and the median fluorescence intensity (MFI) values, reflecting antibody concentrations, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific antibody binding. In case of non-specific binding, this background was subtracted from the MFI values. Human pooled serum was used as a standard.

Statistics

Differences in antibody binding (Luminex[®]) between furunculosis patients and healthy controls were assessed using the Mann-Whitney U test in conjunction with the Benjamini-Hochberg false discovery rate (FDR) multiple testing correction (FDR < 0.05).

Results

Clinical outcome of autovaccination

To assess the impact of autovaccination on the severity and frequency of furunculosis, clinical records were screened and telephone interviews using a questionnaire were performed by a physician (Table 1). All four patients suffered from chronic (7293, 7510, and 9105) or recurrent (6466) furunculosis, which required repeated medical interventions.

Autovaccination reduced the frequency and severity of furunculosis or resulted in complete remission (Table 1). Two patients (7293 and 9105) were free of symptoms after autovaccination treatment. The other two (6466, 7510) still developed single, self-healing furuncles, which did not require medical treatment. All patients reported subjective improvement (Table 1). Side effects occasionally observed by the patients were pain and inflammation at the side of injection and elevated temperature (< 38°C) for two days after injection.

Molecular characterization of the furunculosis strains

The infecting *S. aureus* strains were repeatedly isolated from infected skin lesions. In parallel, nasal swabs were taken to determine the nasal colonization status. The infecting and colonizing *S. aureus* isolates were characterized by *spa* genotyping. Additionally, a panel of virulence genes, including *pvl*, *mecA*, exfoliative toxins and superantigens was detected by multiplex PCR (Table 2).

Three patients were nasal *S. aureus* carriers. Molecular characterization showed that all *S. aureus* furunculosis and nasal strains isolated from one patient over time belonged to the same *S. aureus* clone. Notably, two patients (7293 and 9105) were infected with a community-acquired methicillin-resistant *S. aureus* strain (CA-MRSA), which belonged to the clonal cluster CC59. Both strains harbored the characteristic *mecA* and *pvl* genes and displayed a macrolide-lincosamide-streptogramin B resistance (MLS_B) phenotype. Nasal and infecting strains from patient 6466 were *pvl*-positive and belonged to CC121, a lineage strongly associated with furunculosis [28]. Patient 7510 carried a *pvl*-negative CC8 isolate (Table 2).

Anti-staphylococcal serum antibodies before autovaccination

Taking into account the pronounced variability of the species *S. aureus* we used a personalized approach to study the kinetics of the human antibody response in autovaccination. Two-dimensional immunoblots (2D-IB) served to analyze the binding of each patient's serum antibodies to the antigen spectra of the corresponding infecting *S. aureus* strain. Our analyses were focused on the more informative pH range of 6–11, where most extracellular proteins resolve, while protein A is excluded, so that unspecific IgG binding could be avoided [25].

The furunculosis patients showed a strong antibody response against the extracellular proteins of their infecting *S. aureus* strain already before autovaccination treatment (Fig. 1a). All four patients showed a relatively complex

Table 2 Virulence genes and genotype of *S. aureus* strains

Clinical data				Virulence genes ^a						Genotype		
Patient	<i>S. aureus</i> isolate	Date	Source	<i>mecA</i>	<i>pvl</i>	non- <i>egc</i> SAgs	<i>egc</i> SAgs	<i>eta</i> , <i>etd</i>	<i>agr</i>	<i>spa</i> type	Deduced MLST CC ^b	
6466	6466/1	22.06.07	Pus ^c	–	+	–	<i>g i m n o u</i>	–	4	t645	CC121	
6466	6466/2	20.08.07	Nose	–	+	–	<i>g i m n o u</i>	–	4	t645	CC121	
6466	6466/2a	14.11.07	Nose	–	+	–	<i>g i m n o u</i>	–	4	t645	CC121	
6466	6466/2b	17.07.08	Nose	–	+	–	<i>g i m n o u</i>	–	4	t645	CC121	
7293	7293/1	20.09.06	Pus ^c	+	+	–	–	–	1	t437	CC59	
7293	7293/2	20.09.06	Nose	+	+	–	–	–	1	t437	CC59	
7293	7293/2a	12.12.06	Nose	+	+	–	–	–	1	t437	CC59	
7510	7510/1	27.09.06	Pus ^c	–	–	–	<i>g i m n o u</i>	–	4	t037	CC8	
7510	7510/2	27.09.06	Nose	–	–	–	<i>g i m n o u</i>	–	4	t037	CC8	
7510	7510/1b	20.12.06	Pus	–	–	–	<i>g i m n o u</i>	–	4	t037	CC8	
9105	9105/1	20.11.06	Pus ^c	+	+	<i>b k q</i>	–	–	1	t437	CC59	

^aStaphylococcal enterotoxins (SEs) are indicated by single letters (*a* = *sea*, etc.). *tst* = toxic shock syndrome toxin, *egc* = enterotoxin gene cluster, *eta*, *etd* = exfoliative toxins a and d, *agr* = accessory gene regulator, *pvl* = Panton-Valentine leucocidine (*lukPV*).

^bMLST CC nomenclature was deduced from *spa* types using the Ridom SpaServer database.

^cThe strain isolated from purulent furuncles was used to prepare the autovaccine

antibody binding pattern, especially in the basic pH range. However, there were also numerous protein spots which were not recognized by serum IgG. IB images from pH 4–7 were less complex and a number of spots were due to IgG binding to protein A. Overall, they corroborate the observations made in pH range 6–11 (data not shown).

Next, Luminex® technology was used to compare the antibody response in furunculosis patients (before autovaccination) and healthy controls. Anti-staphylococcal IgG, IgA and IgM serum concentrations in the four investigated patients were comparable to those in healthy individuals ($n=11$) (Fig. 2). Serum IgG and IgA from furunculosis patients reacted with a broad range of staphylococcal

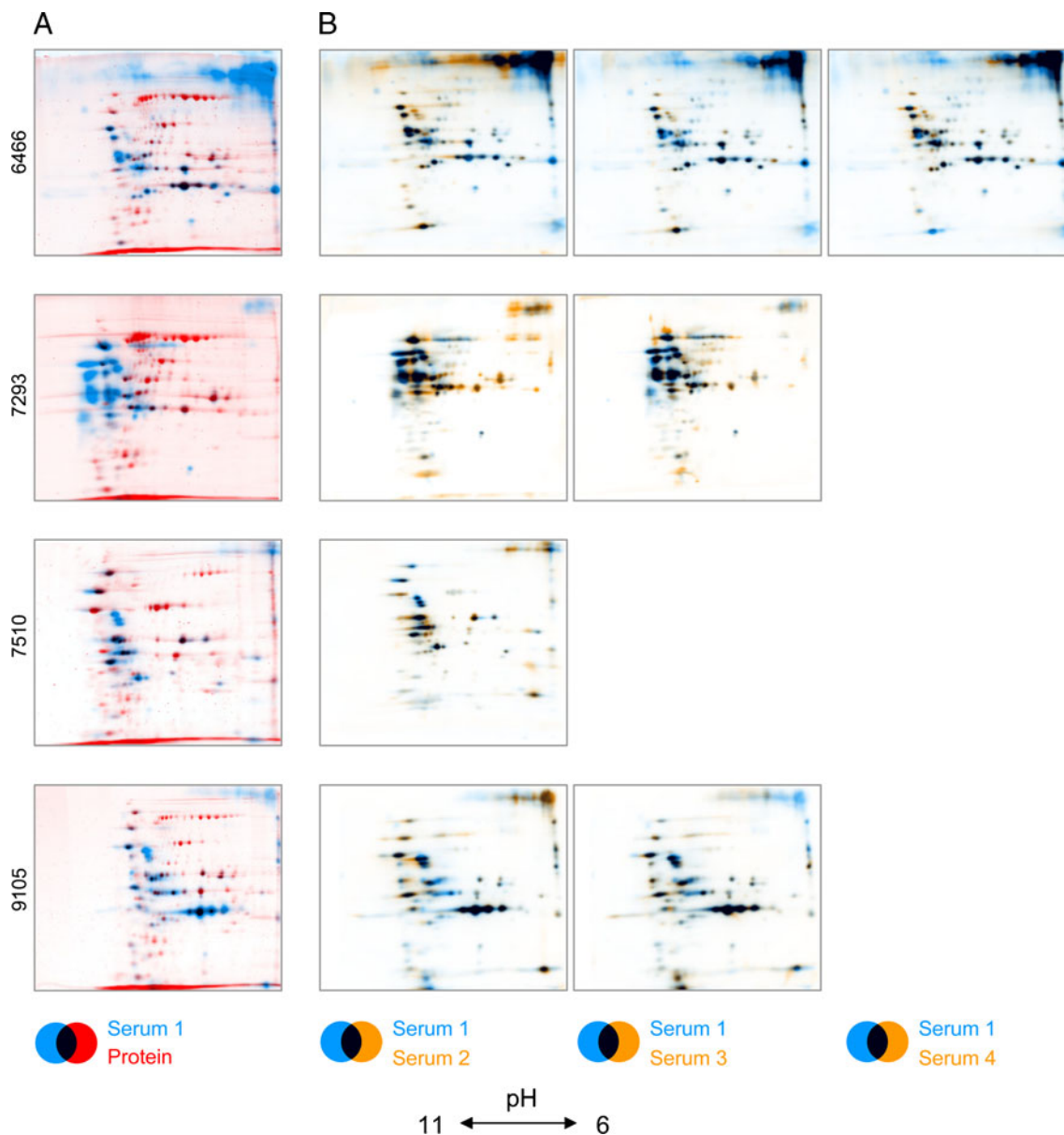


Fig. 1 Serum IgG response to extracellular *S. aureus* proteins in autovaccination. **a** Overlay images of Flamingo®-stained 2D gels and corresponding 2D-immunoblots. The proteins released during post-exponential growth by the four invasive *S. aureus* strains were separated by 2D gel electrophoresis (pH 6–11) and stained with the fluorescence protein dye Flamingo® (red). The four bacterial isolates differed in their secretomes. In parallel, the extracellular bacterial antigens were blotted onto PVDF membranes and incubated with the corresponding patients' sera obtained before autovaccination. The overlay images (protein – red; IgG binding – blue) showed a strong

antibody response to the infecting strain already before autovaccination. **b** Kinetics of individual IgG responses to autovaccination. Extracellular antigens from the infecting *S. aureus* strains were separated by 2D gel electrophoresis and quantitative IBs were performed with the corresponding patients' sera obtained at different time points. Overlays of 2D-IBs with sera obtained before (blue) and during (orange) autovaccination. Autovaccination induced a moderate boost in the IgG response. One out of the three technical replicates is shown

surface antigens, toxins (e.g. PVL, superantigens) and immunomodulatory proteins. There was little IgM binding to staphylococcal antigens in controls as well as two patients, but strong binding to many *S. aureus* antigens in the other two patients (Fig. 2). Superantigens encoded by the enterotoxin gene cluster, *egc*, were on average bound less strongly than others. To conclude, furunculosis patients did not have a general lack of anti-staphylococcal antibodies.

Antibody response during autovaccination

To test whether autovaccination triggers an antibody response, we studied the kinetics of antibody binding using 2D-IBs and Luminex. First, we performed 2D-IBs with sera obtained over

the course of autovaccination (2–4 samples). Superimposition of 2D-IB images from serum samples obtained before autovaccination (blue) and later (orange) showed almost identical antibody binding patterns as indicated by the dominance of black spots on the superimposed images (Fig. 1b). Only a few new IgG-binding specificities, indicated by purely orange spots, were generated. Patient 7293 was exceptional, because we observed a boost in the IgG-binding as well as numerous novel IB-spots.

Next, we quantified the individual IB-spot intensities and determined the changes in antibody binding during autovaccination (Fig. 3). The patients' sera obtained before autovaccination reacted with 55–82 protein spots. Patient 7293 showed the weakest antibody binding before vac-

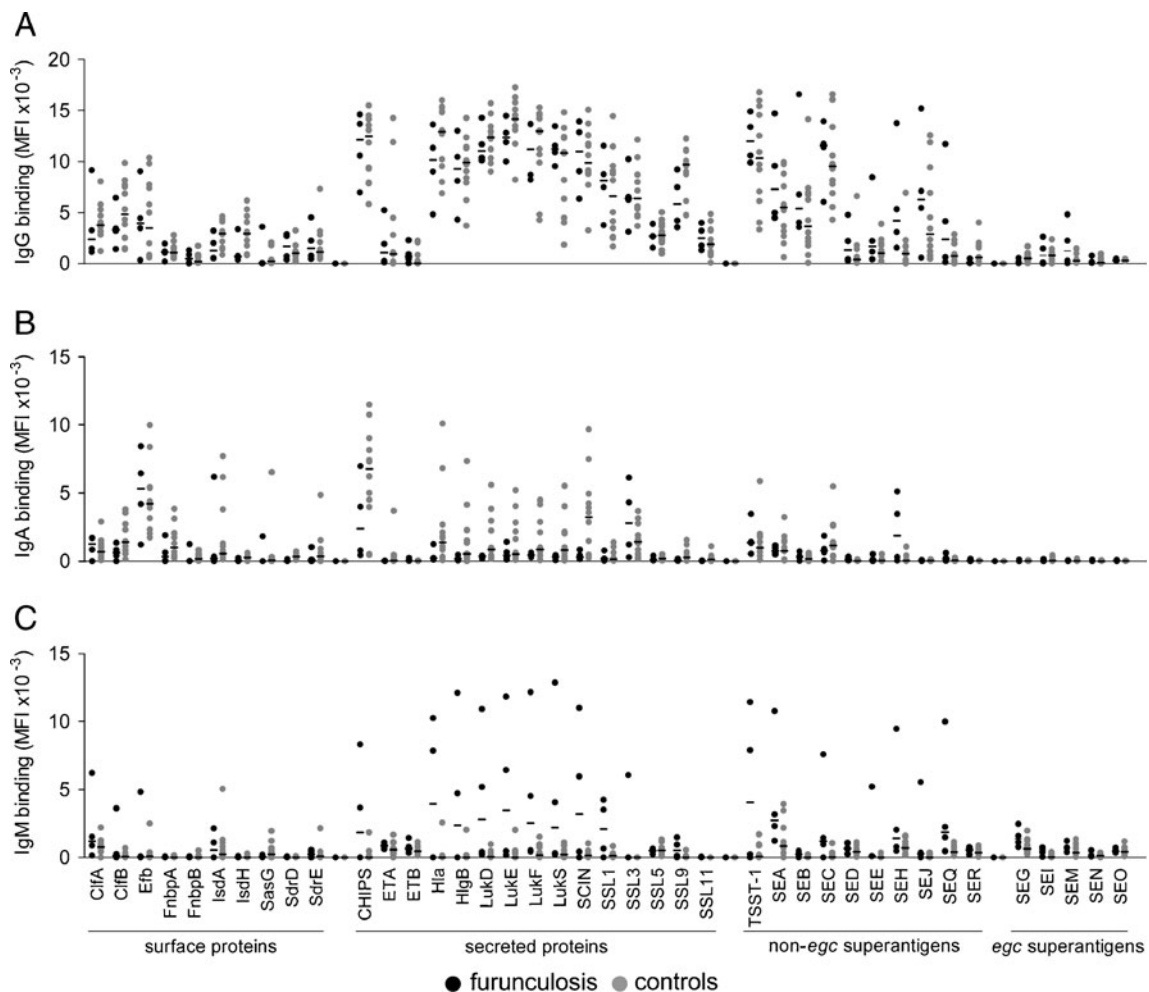


Fig. 2 Serum IgG, IgA and IgM response to *S. aureus* surface proteins and toxins in furunculosis patients and healthy controls. Serum IgG (a), IgA (b) and IgM (c) antibody binding to a panel of 40 *S. aureus* antigens was assessed with a Luminex assay and Ig binding to individual antigens is depicted as median fluorescence intensity (MFI). Each symbol represents a single individual. Median levels of anti-staphylococcal antibodies for patients ($n=4$; before autovaccination) and healthy controls ($n=11$) are indicated. The antibody binding patterns of furunculosis patients and healthy controls were not

significantly different (Mann-Whitney U test using the Benjamini-Hochberg false discovery rate multiple testing correction). Abbreviations: *ClfA* Clumping factor A, *Efb* extracellular fibrinogen-binding protein, *FnbpA* Fibronectin binding protein A, *IsdA* iron-responsive surface determinant, *Sas* *S. aureus* surface protein, *Sdr* serine-aspartate dipeptide repeat proteins, *CHIPS* chemotaxis inhibitory protein of *S. aureus*, *Luk* leukocidin, *SCIN* staphylococcal complement inhibitor, *SSL* staphylococcal superantigen-like protein, *SE* staphylococcal enterotoxin

nation (Fig. 3a). In this patient, vaccination induced a two- to five-fold intensity increase in most spots (59/67) (Fig. 3b). A moderate boost was observed in patient 7510 with 22/83 spots increasing more than two-fold. The antibody patterns of the other two patients (6466 and 9105) remained almost unchanged (Fig. 3b). Notably, autovaccination induced only few novel antibody specificities. In conclusion, autovaccination boosted the IgG response to extracellular antigens in two patients, while the antibody response of the other two patients was not affected.

Finally, we applied the Luminex assay to test whether autovaccines trigger an antibody response to surface-bound staphylococcal antigens. Indeed, three out of four patients showed a more than two-fold increase in IgG binding to surface antigens, such as ClfA (7293, 7510, 9105), ClfB (9105), SdrD (7510) and SdrE (7293) (Fig. 4). Similarly, we observed a boost in the IgA response against surface proteins (suppl. Fig. 1). Thus, vaccination induced a moderate boost of the antibody response against staphylococcal surface proteins. Novel antibody specificities against surface proteins were not generated.

Discussion

Autovaccines have been applied in chronic staphylococcal infections for decades, but their mechanism of action is still unknown. In this pilot study, we demonstrate that autovaccination only slightly boosts the antibody response to extracellular bacterial antigens and surface proteins.

This pilot study was not designed to test the efficacy of autovaccination, but rather to characterize the kinetics of the antibody responses. Therefore, we analyzed only a small cohort and did not include placebo controls. Moreover, some clinical outcome criteria (severity score, rating of clinical improvement) are subjective, which limits the evaluation of the clinical outcome. Nevertheless, it is interesting to note that based on the applied criteria all four patients, two of whom were chronically infected with CA-MRSA, clinically improved during treatment. In the 1970s, Ring et al. reported that oral autovaccination treatment improved the clinical state in 11/18 patients with chronic posttraumatic osteomyelitis [15]. Similarly, a controlled study of 292 children with paranasal sinusitis demonstrated that antibiotic therapy plus autovaccine was superior to antibiotic therapy alone [29]. Overall, little is published in English on the efficacy and safety of autovaccination. In particular, prospective, controlled, randomized, and, if possible, (double)-blind clinical studies are lacking.

Already before autovaccination, the study patients had serum antibodies that bound to a broad antigen spectrum of their infecting *S. aureus* strain. There was no obvious defect in their anti-staphylococcal antibody response. Furunculosis patients suffer from chronic or recurrent infection for many months or even years and, therefore, the immune system is frequently and intensively exposed to the invasive strain and establishes a memory response. Moreover, most furunculosis patients are *S. aureus* carriers and the nasal and infecting strains are usually identical

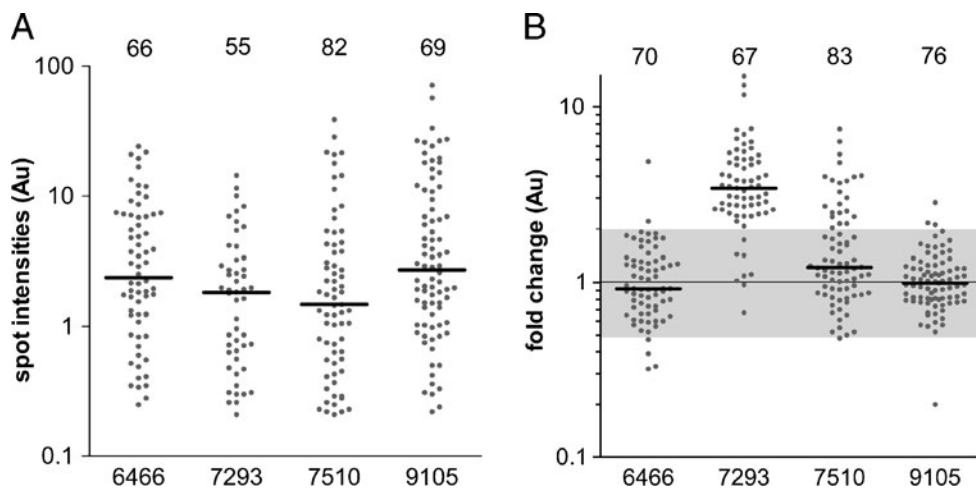


Fig. 3 Quantification of IgG binding to extracellular *S. aureus* antigens. Spot intensities on 2D-IB were determined from three or more replicate blots and median intensities in arbitrary units (AU) are depicted. The threshold was set at a volume of 0.2 AU. **a** Spot intensities before autovaccination. Before autovaccination, we detected strong serum IgG binding to numerous antigens from the infecting strain. The total number of immunoblot spots is displayed above each data set. Median intensities from all spots are indicated. **b**

Changes of spot intensities during autovaccination. To monitor changes in IgG binding, the ratios between the last and first serum sample were calculated for each spot (ratios of median spot intensities). In patient 7293 most spots showed a two- to five-fold increase in IgG binding. Shaded area corresponds to a two-fold change. The total number of immunoblot spots obtained with the last serum is displayed above each data set. The overall medians are depicted

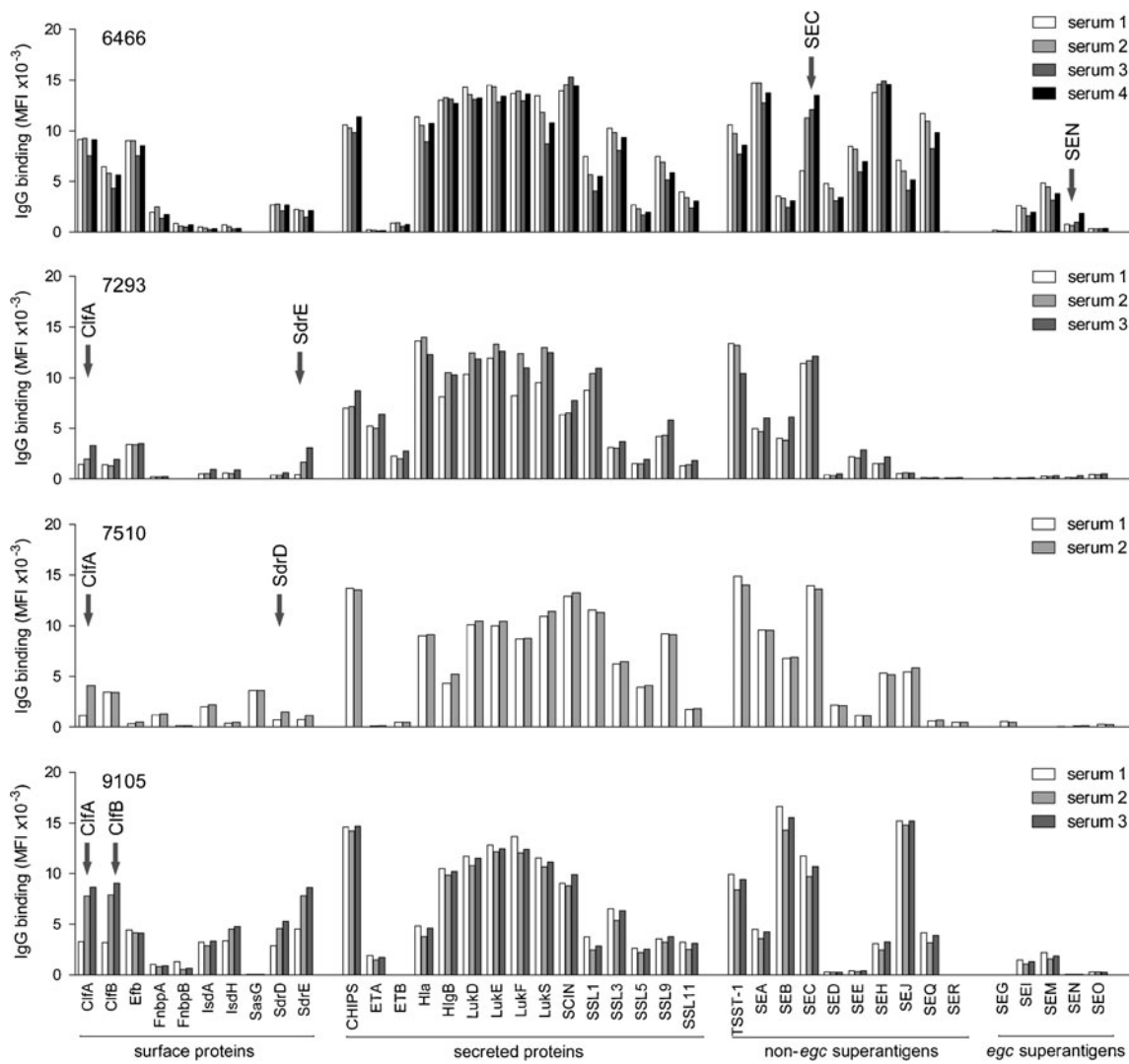


Fig. 4 Kinetics of the serum IgG response to *S. aureus* surface proteins and toxins over the course of autovaccination. IgG binding to a panel of 40 *S. aureus* antigens was assessed with a Luminex assay. Each graph represents a single patient. Per patient, the level of

antigen-specific antibodies in the time course of autovaccination is shown. *Arrows* indicate a more than two-fold increase in antibody binding. See legend to Fig. 2 for more detailed information on the antigens

[28]. In this study, three out of four patients were colonized with the infecting strain in the nose. Thus, the nose appears to be a potential source for repeated endogenous infections. In agreement with our findings of a robust baseline anti-*S. aureus* antibody response in the furunculosis patients, we previously reported that healthy human carriers are pre-immunized with their colonizing strain [12, 25].

By applying 2D-IB and Luminex assays we found that autovaccination only slightly boosted the pre-existing antibody response to secreted bacterial antigens and surface proteins. Notably, autovaccination did not induce a profound de novo generation of antibody specificities. These findings suggest that repeated subcutaneous immunization

does not strongly trigger the humoral immune response in these well pre-immunized patients. In line with this, Szkaradkiewicz et al. analyzed the IgG response in patients treated with autovaccines using 1D-immunoblots and also observed no changes in IgG binding patterns to *S. aureus* polypeptides [30]. In contrast to autovaccination of furunculosis patients, systemic *S. aureus* infection strongly enhances the anti-staphylococcal antibody response in patients [10].

We previously reported a high degree of strain-specificity in the anti-*S. aureus* antibody response [12]. However, it is also possible that some antibody specificities are cross-reactive. For example, patient 6466 mounted an antibody response against SEC, but the strain used for

autovaccination lacked the *sec* gene. SEC belongs to a family of 21 staphylococcal superantigens with up to 90% sequence homology, which could explain the observed cross-reactivity [31, 32].

The pore-forming toxin PVL is epidemiologically strongly linked to *S. aureus* furunculosis [3, 28]. In this study, three out of four patients were infected with *pvl*-positive strains. All patients had high levels of IgG antibodies against both subunits of the toxin, LukF and LukS, which did not increase during autovaccination.

Little is known about the mechanisms by which autovaccines might influence the disease course or impact on the immune response. Halasa et al. reported that autovaccination increases the concentration of *S. aureus* strain-specific agglutinating antibodies and enhances the phagocytosis of bacteria by peripheral blood granulocytes [13]. Similarly, clinical improvement of acne after autovaccination with killed *Propionibacterium acnes* was accompanied by the generation of specific antibodies against bacterial structures [33]. Still, it is difficult to imagine that the observed moderate changes of antibody titers should fully explain the clinical effects of the treatment. Other groups have reported that autovaccination down-regulates Th1 cell function and reduces delayed type hypersensitivity reactions [15, 21, 34]. In conjunction with the observation that T cells can influence the recruitment and anti-bacterial action of granulocytes [35, 36], this opens an interesting perspective for research. Moreover, the humoral immune response to non-protein staphylococcal antigens, such as lipoteichoic acid, wall teichoic acid and capsular polysaccharides, might be clinically important and will be addressed in future analyses [11, 37]. Finally, we cannot rule out that formalin-fixation of the vaccine preparation influences the three-dimensional antigen structure, which might influence the antibody response, especially to surface antigens.

The rapid spread of multiresistant *S. aureus* strains enforces the development of new strategies to combat staphylococcal infections. In view of the pronounced variability of the bacterial species, personalized approaches could hold promise for patients with recurrent or chronic *S. aureus* infection. Autovaccination could serve as a model that merits further investigation.

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