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Potential targets for immunotherapy and infection imaging on the cell surface of Staphylococcus aureus

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Chapter 7

General summarizing discussion

The intimate relationship between humans and the pathogen *Staphylococcus aureus* dates back a long time in evolution. The earliest description of *S. aureus* was recorded in 1882 by the Scottish surgeon Sir Alexander Ogston. At the time, he described *S. aureus* cells detected in the pus of acute abscesses as micrococci growing sometimes in clusters and sometimes in chains [1]. The close and long-term relationship between *S. aureus* and the human host is clearly reflected by the staphylococcal virulence factors that specifically target human immune responses and that have no or limited activity in other organisms. These include the Panton-Valentine Leukocidin (PVL)[2], γ-hemolysin CB (HlgCB)[3], Enterotoxin A (SEA) [4], the staphylococcal complement inhibitor (SCIN) [5], the chemotaxis inhibitory protein (CHIPS) [6] and the staphylokinase (Sak) [7]. The intimate relationship between *S. aureus* and the human host is also mirrored by the ubiquitous presence of *S. aureus* in humans. In the large majority of cases, the *S. aureus* carriage is asymptomatic but, when the primary human defenses are breached, *S. aureus* is capable of colonizing and infecting virtually any site of the human body, inside [8] and outside [9]. Unfortunately, the long co-existence has also taught *S. aureus* how to effectively evade the human immune defenses, resulting in very high mortality rates from *S. aureus* bacteremia before the introduction of antibiotic therapy [10]. The recent alarming increase of antibiotic resistance in *S. aureus*, and especially the rise of methicillin-resistant *S. aureus* (MRSA), now threatens to bring back the old-time high mortality rates. Consequently, there is a pressing need to develop novel prophylactic or therapeutic treatments against *S. aureus* infections as alternatives to antibiotics therapy [11,12].

This thesis reports on human antibody responses to surface-exposed *S. aureus* proteins, and possible diagnostic applications of anti-staphylococcal monoclonal antibodies. As previous active immunization efforts have failed to bring an effective *S. aureus* vaccine to the clinic, it is very important to better understand the host-pathogen interactions that contribute to vaccine failure, the success of *S. aureus* as a pathogen, and its effective escape from immune control [13,14]. Most humans are exposed to *S. aureus* early in life: more than 70% of newborn babies have at least one positive nasal culture with *S aureus* [15]. Later on, about a third of the healthy adult individuals are *S. aureus* carriers [9,16]. Underneath this high prevalence of *S. aureus* on the human body lies a highly diverse immune response, the shape of which is influenced by exposure history, differences in the *S. aureus* strains that have been encountered, genetic determinants of the host, and environmental factors [9,16–21]. Despite the fact that anti-staphylococcal antibody levels increase strongly during bacteremia [16,19], the *S. aureus*-induced immune response still fails to provide significant protection against subsequent infections. However, it is possible that a *continuous* exposure to staphylococcal antigens might improve the effectiveness of the immune response [17]. Remarkably, *S. aureus* bacteremia is infrequently observed in older patients with the blistering disease epidermolysis bullosa (EB), despite high *S. aureus* colonization rates in their (chronic) wounds. This suggests that the high anti-staphylococcal IgG levels in these patients, due to continuous exposure to multiple strains, may be protective against invasive *S. aureus* infections [22–26]. If so, this would argue that active immunization of healthy individuals with appropriate antigens may indeed elicit an immune response similar to the one observed in EB patients that provides effective protection against *S. aureus* infections. Several studies, including the ones described in this thesis, have already studied the EB patients' immune responses to evaluate the antigenicity of several *S. aureus* proteins [23,26]. The importance of these patients' immune responses to *S. aureus* was the incentive for the research described in all experimental chapters of this thesis. Chapter 2 describes the immune responses against surface-exposed *S. aureus* proteins, indicating their potential for use as vaccine antigens. Chapter 3 exemplifies the use of plasma from an EB patient for rapid antigen screening. Chapter 4 reports on a newly identified human monoclonal antibody (humAb) cloned form B-cells donated by an EB patient, which binds to the *S. aureus* SCIN protein. In Chapter 5, EB patient IgGs and the recently described

anti-IsaA humAb 1D9 are shown to preferentially bind the N-terminus of the immunodominant staphylococcal antigen A (IsaA). Interestingly, 1D9 was found to target a 62 amino acid N-terminal region of the mature IsaA protein. Finally, in Chapter 6 the application potential of 1D9 in the *in vivo* detection of *S. aureus* infections is highlighted.

Surface-exposed and secreted proteins of *S. aureus* have previously been studied as potential vaccine targets [27,28]. While most studies focused on covalently cell wall-anchored proteins of *S. aureus*, less-studied non-covalently cell wall-bound proteins are also promising candidate vaccine targets [29], with two of these proteins, IsaA and Atl, being the most often found surface-associated proteins (Chapter 1). Using a combined bioinformatics and proteomics approach, a set of non-covalently cell wall-bound proteins was identified, which included both newly identified and previously reported surface proteins. Subsequently, the antibody responses in plasma from selected EB patients and healthy volunteers were measured (Chapter 2). This led to several interesting observations. 1) For all tested non-covalently cell wall-bound proteins, on average, significantly higher antibody responses were observed in plasma samples from EB patients compared to plasma samples from the healthy carrier controls. The highest antibody levels were observed when patients were colonized by multiple *S. aureus* strains. 2) There was a large inter-patient variation in the levels of antibodies against particular non-covalently cell wall-bound proteins. 3) There was large variation in the antibody responses from the same EB patient against different antigens. In other words, while all EB patients showed high immune responses against non-covalently cell wall-bound proteins, there was no individual antigen with a high immune response in all patients, and there were no patient sera with a consistently high antibody titer to all antigens. Of note, only a small portion of all potential non-covalently cell wall-bound proteins have been studied for potential human antibody responses. Further research should therefore help to elucidate the group of antigens that can elicit consistently high immune responses in a majority of EB patients. This patient group is of particular interest, as their immune responses to *S. aureus* can potentially uncover antibody profiles that may guide approaches for eliciting protective immune responses by active immunization (Figure 1).

To probe the immune response of EB patients against a specific antigen, it is usually necessary to first generate the antigen in purified form. Chapter 3 presents an expanded set of *Lactococcus lactis* vectors that facilitates the production and purification of differentially tagged and secreted proteins from the culture medium. Proteins tagged with the *Strep*-tag can be affinity-purified or immobilized in one step. The Avi- and His_6-tag constructs enable the production and purification of proteins that have been site-specifically labeled with biotin. Expression of *strep*-tag labeled and secreted *L. lactis* proteins has not been described before. The addition of this third-generation vector set to the currently available arsenal for cloning and expression of *S. aureus* antigens will, most likely, be useful for future research efforts.

Several non-covalently cell wall-bound proteins of *S. aureus* were identified *in vitro* (Chapter 2). However, it is possible that certain secreted proteins of *S. aureus* also behave as non-covalently cell wall-bound proteins, but only under specific conditions. This is exemplified by the SCIN protein, which is usually found in the supernatant of *S. aureus* cultures. Accordingly, SCIN is generally regarded as an extracellular protein [5]. However, SCIN behaves as a non-covalently cell wall-bound protein in the presence of human plasma (Chapter 4). This relates to its recruitment to staphylococcal cells by binding of C3 convertases. It is thus very well possible that the number of non-covalently cell wall-bound proteins of *S. aureus* is actually substantially larger under *in vivo* conditions than currently believed on the basis of *in vitro* culturing data. This reignites interest in the proteomics analysis of the *S. aureus* ‘corona’, which is the human and staphylococcal protein complement that is

recruited to the *S. aureus* cell surface *in vivo*. The composition of the corona can be approximated by *in vitro* incubation of *S. aureus* cells in human body fluids, such as serum[30]. Of note, the studies described in Chapter 4 made use of the anti-SCIN humAb 6D4, which was shown to inhibit the activity of SCIN by binding to its active site. Thus, 6D4 can be added to a growing list of specific anti-staphylococcal antibodies that may find use in future clinical applications. As SCIN is not essential for the virulence of *S. aureus*, an application in passive immunization is less likely, but 6D4 may have potential for infection imaging as shown with another anti-staphylococcal humAb (1D9) in Chapter 6 of this thesis.

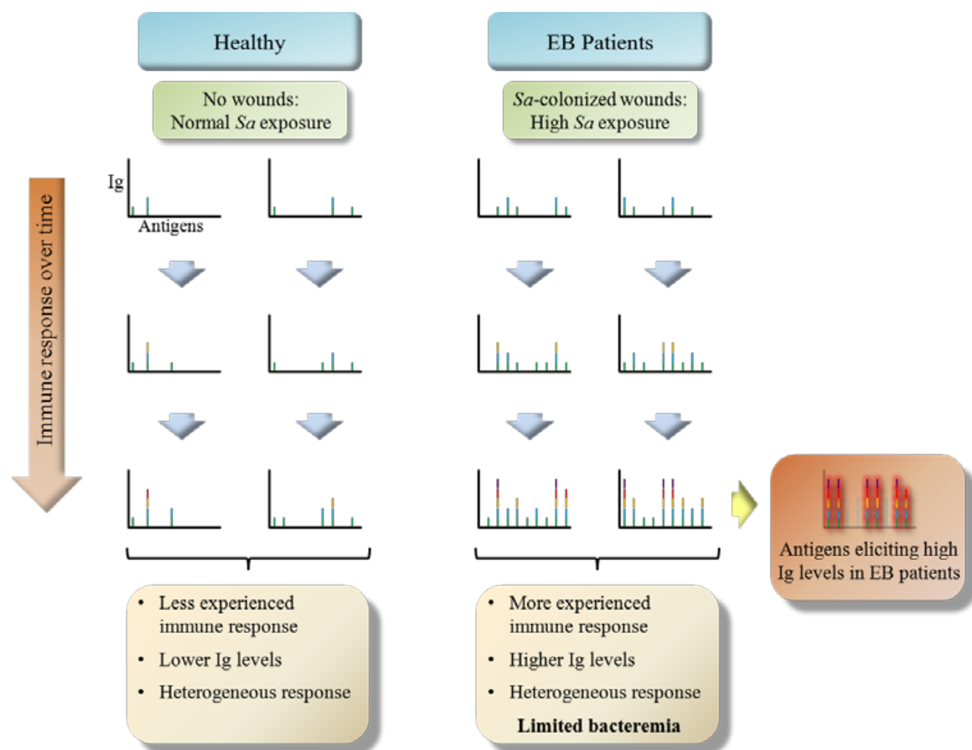

Figure 1: Schematic representation of the development of humoral immunoglobulin responses against *S. aureus* throughout life of healthy individuals vs. EB patients

From an early age, *S. aureus* (*Sa*) exposure drives a heterogeneous immune response in healthy individuals, which is characterized by relatively low anti-*Sa* immunoglobulin (Ig) levels. Due to their chronic wounds colonized with *Sa* and potentially recurrent bacteremia in early age, EB patients are subject to high *Sa* exposure, which drives a still heterogeneous immune response characterized by high overall anti-*Sa* levels to a plethora of staphylococcal virulence factors. During life, EB patients undergo severe *Sa* exposure, which elicits stronger immune responses that are reflected in high Ig levels against specific *Sa* antigens. Identification of the antigens that elicit high Ig levels in several EB patients provides a picture of potentially effective EB patient antibody responses.

Passive immunization, the transfer of active humoral immunity in the form of ready-made antibodies for the treatment or prevention of infectious diseases, has been used for over a century [31]. Previous reports have shown that both the recently identified anti-IsaA humAb 1D9, and another humanized monoclonal anti-IsaA antibody, improved the survival of mice challenged with *S. aureus* infections by passive immunization [32,33]. However, active immunization of mice with an octa-valent staphylococcal vaccine containing IsaA did not result in protection against this pathogen, despite

generating a high IgG response [34]. This difference may be explained by preferred binding of anti-IsaA IgGs in IsaA-immunized mice to the C-terminus of IsaA, while the partially protective IsaA-specific humAb 1D9 binds to the N-terminus of IsaA (Chapter 5). Intriguingly, also IgGs in plasma samples from EB patients were found to preferentially bind the N-terminal moiety of IsaA. Apart from the fact that this differentiated epitope recognition may just reflect that a murine immune response is not identical to the human immune response, these findings suggest that the initial immune response against an *S. aureus* antigen may not be the most efficient response. Instead, repeated exposure, as occurs naturally in EB patients due to high *S. aureus* colonization rates, may yield a more efficient immune response. Alternatively, the N-terminal domain of IsaA may not be properly presented to the immune system if the purified IsaA protein is used for immunization. Thus, it is conceivable that immunization only with the antigenic N-terminal region may be associated with a better outcome, perhaps also without repeated exposure. In addition, the observations presented in Chapter 5 suggest that future research could benefit from analyzing functional fragments of particular antigens instead of the full size proteins. This is exemplified in Chapter 2 for the Atl protein, where two functional fragments designated as Atl1 and Atl2 were separately expressed and used for screening the human IgG response. Such an approach will indicate the preferred (if any) binding sites of potentially protective IgGs in EB patient sera.

Physical removal of infection foci may be necessary to fight staphylococcal infections. For example, this is the case for infections precipitated on implanted materials and medical devices [35]. Several examples of imaging probes with broad specificity for bacteria have been published. Yet, relatively few studies have reported the targeted imaging of infections by a particular bacterial species [36]. Chapter 6 describes the use of the humAb 1D9 labeled with the infrared fluorophore IRDye 800CW as a specific *Staphylococcus*-targeted imaging probe. Detection of the infrared signal crossing the human skin was shown, in agreement with the previously reported penetration depth of ~1 cm [37], as well as significant specific detection of *S. aureus* in a murine skin infection model. As has been demonstrated previously for oncological applications [38–40], the use of labeled antibodies specific for *S. aureus* antigens as probes for targeted fluorescent imaging could potentially guide and improve the surgical removal of infection foci, by specifically pinpointing the infected tissue or implant to be removed, while sparing the healthy surrounding tissue. While the use of 1D9 labeled with IRDye 800CW or the positron emission tomography tracer ^{89}Zirconium was enough for specific detection of *S. aureus* in murine infection models, it is conceivable that the simultaneous use of two or more *Staphylococcus*-specific antibodies, like 1D9 and 6D4, can drastically increase specificity, sensitivity and signal-to-background ratios in the detection of *S. aureus* infections. Alternatively, testing of additional tracers [36], for example tracers suitable for optoacoustic imaging[41], could potentially facilitate the detection of infections seated deeper inside the body by increasing the tissue penetration range for detection.

In conclusion, several potential *S. aureus* targets for the development of active or passive immunization approaches have been described, and a pipeline for targeted identification and development of candidate antigens has been devised (Figure 2). The effective anti-staphylococcal immune responses of heavily colonized EB patients, who show infrequent *S. aureus* bacteremia, were used to define the antigenic and therapeutic potential of several specific antigens. In addition, two promising *S. aureus*-specific humAbs, 1D9 and 6D4, have been described. For one of them - 1D9 - it was shown that significant *S. aureus*-specific *in vivo* detection of infection could be achieved in animal models. Future research with animal infection models should show whether these antibodies can be applied for the fight against *S. aureus* infection, either by themselves or in combination.

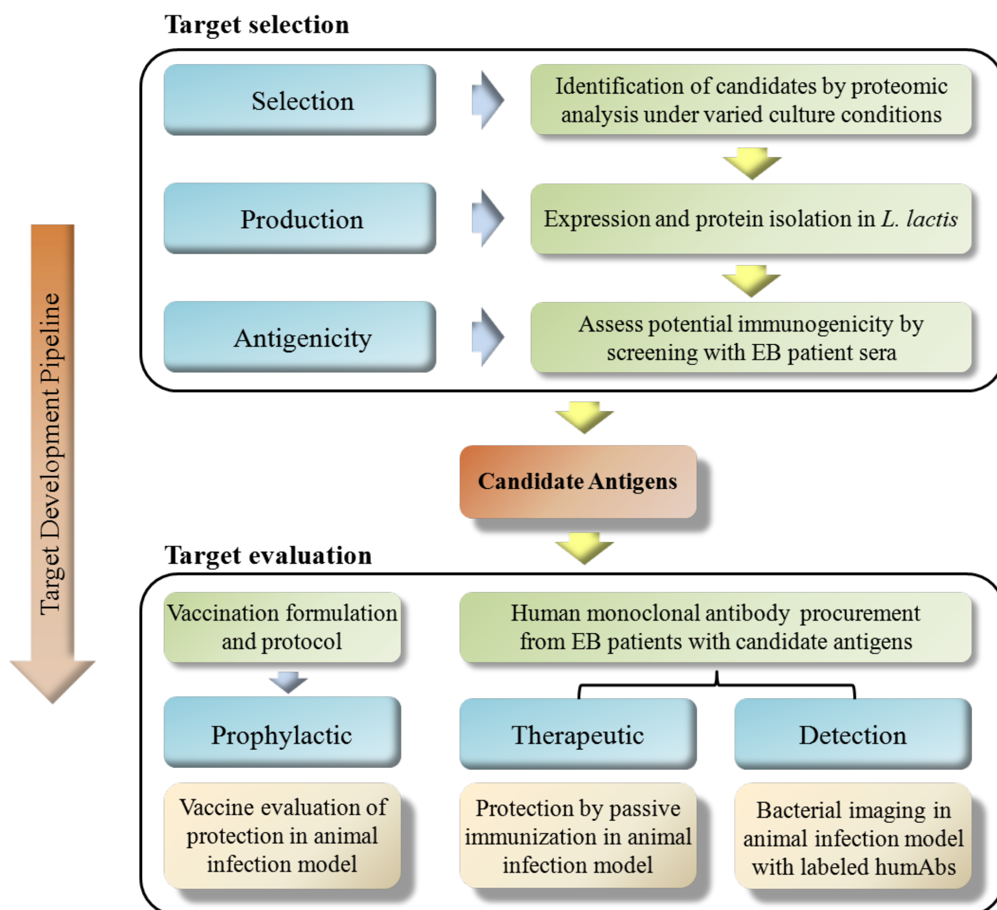

Figure 2: Schematic representation of a pipeline for vaccine target selection and evaluation
Potential antigenic targets are identified from literature mining and by proteomic analysis of different culture fractions from diverse bacterial isolates under culture conditions that approximate conditions in the human host.[42] Once selected, proteinaceous antigens are expressed in *L. lactis*, isolated, and tested for potential human immunogenicity by screening with sera from *S. aureus*-challenged EB patients (i.e. target selection). Selected candidate antigens are then evaluated for efficacy in the protection against infection by active immunization in an animal infection model. Production and selection of human monoclonal antibodies (humAbs) against selected targets can be performed with the help of B-cells from EB patients. Selected humAbs can then be evaluated for their potential prophylactic or therapeutic use through passive immunization studies with animal infection models, and/or for their applicability in imaging of bacterial infections upon labeling with appropriate tracers.

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