Towards the development of antistaphylococcal immunotherapy

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 17-04-2019
Chapter 7

Summary and future perspectives

For the general human population, carriage of the Gram-positive bacterium *Staphylococcus aureus* is not associated with symptoms of disease. In fact, over time about 60% of the population is probably intermittently carrying this bacterium. However, upon invasion of the human body, it becomes evident that *S. aureus* is actually a serious pathogen that can cause many different diseases. Such infections are frequently difficult to treat, due to the emergence and spread of highly antibiotic resistant lineages in hospitals and the community. Therefore, new options for prevention and therapy of staphylococcal infections need to be developed and implemented in clinical practice. Over the past decades, many studies have attempted to develop active or passive immunization approaches with the aim to protect frail individuals against *S. aureus* infections. Although some studies using small laboratory animals were apparently successful, to this date no effective vaccine for human use has successfully passed the stage of clinical trials. It is currently debated why exactly it is so hard to develop effective immunization approaches against *S. aureus*, but it is tempting to speculate that this relates primarily to the fact that, for ethical reasons, early-stage preclinical studies can only be performed in laboratory animals and, secondly, to the types of antigens that have been selected for these preclinical studies in animals. In the studies described in the present thesis, different approaches for active and passive immunization were explored in which it was attempted to overcome the limitations of animal models by comparing immune responses in ‘mice and men’. In addition, a passive immunization approach was explored, which was based on the cloning of a human antibody that recognizes a surface protein of *S. aureus* – the immunodominant staphylococcal antigen A (IsaA) - that is invariantly expressed by this pathogen. Although these investigations have not yet led to prophylactic or therapeutic approaches that are ready for the clinic, several potentially valuable insights were obtained that may pave the way towards the development of antistaphylococcal immunotherapy.
For the studies presented in this thesis, the focus was on a number of peptidoglycan hydrolases as potential targets for active and/or passive immunization. These peptidoglycan hydrolases were selected as potential targets, because various proteomic studies have shown that these (i) enzymes are invariantly expressed by all investigated clinical \textit{S. aureus} isolates, (ii) that they are capable of eliciting an immune response in humans, and (iii) that they have a role in staphylococcal virulence (Chapter 1). Importantly, peptidoglycan hydrolases are intrinsically exposed on the bacterial cell surface, which renders them readily accessible to antibodies produced by the human immune system. This would then lead to opsonization and subsequent clearance of the bacteria by the phagocytic cells of the human immune system. Based on different criteria, especially invariant and high expression, special attention was attributed to the peptidoglycan hydrolases named Atl, LytM and IsaA. However, in addition to these peptidoglycan hydrolases, also several other known virulence factors of \textit{S. aureus}, specifically Nuc and the four phenol-soluble modulins α1-4, which are both immunogenic and invariantly produced by all tested isolates, were included in some of the present analyses. Eventually, IsaA was selected as the main focus of the present studies, because (i) it is the \textit{S. aureus} protein that elicits the highest antibody responses in humans, (ii) it was possible to produce this protein in ample amounts for immunization studies and (iii) it was possible to develop a fully human monoclonal antibody against IsaA.

\textbf{Chapters 2} and \textbf{3} describe a novel pipeline for the expression and purification of tagged surface-exposed and secreted antigens from \textit{S. aureus}. Specifically, this involved the use of the Gram-positive bacterium \textit{Lactococcus lactis} as a host for inducible antigen expression. The development of this host and a set of specific cloning vectors was needed, because it turned out to be difficult to use the frequently applied expression host \textit{Escherichia coli} for this purpose. In doing so, invariant staphylococcal proteins, such as LytM, Aly, SA2097, IsaA, SA0620 and Nuc, were successfully produced and isolated. The \textit{L. lactis} expression system also allowed the addition of various tags to the produced antigens, especially the His\textsubscript{6} and AVI-tags that can be applied for metal affinity purification, biotinylation and localization of the expressed antigens. A comparison between the intracellular or secretory production of the His\textsubscript{6}-
tagged antigens showed that, with the exception of SA0620, higher yields were obtained when the antigens were secreted. This was achieved by fusing the antigens to the signal peptide of the major secreted protein Usp45 of *L. lactis*. Further improvements were achieved by deleting the gene for the extracellular protease HtrA of *L. lactis*, which reduced product degradation, and by deleting the gene for the autolysin AcmA, which reduced cell lysis and the shedding of cellular components into the growth medium. Thus, by introducing merely two mutations it became much easier to purify the secreted His$_6$-tagged staphylococcal antigens and, in fact, this was possible in a one-step procedure. Importantly, the purified LytM, Aly, Nuc, ClfB and SagA (i.e. SA2100) proteins retained their biological activity, indicating that these heterologously produced proteins were correctly folded. As shown in Chapter 3 for the *S. aureus* IsdB protein, even a post-translational modification such as phosphorylation is conserved in *L. lactis*, albeit that the heterologously produced IsdB was phosphorylated on an alternative Tyr residue.

Even though it can be concluded that the developed *L. lactis* expression system as described in Chapters 2 and 3 is highly suited for the production of staphylococcal antigens, there is still some room for further improvements. In the first place, despite the deletion of the *L. lactis* htrA gene, there was still some degradation detectable for antigens such as SA0620, SA2100 and pro-Atl (Chapter 3). Fortunately, this was overcome by moving the His$_6$-tag from the C-terminus to the N-terminus of the heterologously produced antigens. At present, it is not known which proteases are responsible for this residual degradative activity and it is even not clear whether it occurs in the cytoplasm, membrane or cell wall of *L. lactis*. Clearly, this will require further detailed studies, possibly involving the elimination of known quality control proteases, such as ClpP or FtsH. A second potential problem is the observed incomplete secretion of certain staphylococcal antigens, such as SA0620. This may relate to possible incompatibility with the Sec machinery of *L. lactis*, the presence of hydrophobic or charged residues exposed on the protein surface, or the presence of particular cell wall-binding domains. Such features might preclude effective membrane translocation, or they might cause retention of a staphylococcal antigen in the membrane or cell wall of *L. lactis*, thereby setting limits to effective secretory production.
To assess whether it is possible to vaccinate against *S. aureus* infections by using invariantly produced cell surface-exposed antigens of this pathogen, an octa-valent antigen mixture was created as described in Chapter 4. This antigen mixture consisted of the IsaA, pro-Atl, LytM and Nuc proteins produced in *L. lactis* plus the four chemically synthesized phenol-soluble modulins α1-4 (PSMα1-4). The PSMs were chemically synthesized because they are relatively small peptides and, at the time of the study, it was not yet known how exactly they are secreted by *S. aureus*. Of note, sera from patients with the genetic blistering disease epidermolysis bullosa (EB) contained high IgG levels against all eight included antigens, which is probably due to the fact that these patients are continuously challenged with *S. aureus* as their chronic wounds are heavily colonized by different lineages of this pathogen. This suggested that a mixture of these eight antigens might be a good candidate vaccine. Indeed, subcutaneous immunization of BALB/cBYJ mice led to high IgG responses against the eight applied antigens. However, the produced antibodies did not protect mice against *S. aureus* bacteremia or skin infections. There are several possible reasons for this lack of protection conceivable, including the applied animal study design, the role of antibodies in the protection against staphylococcal infections, or the antigens that were used for immunization. An important factor might be that, during an *S. aureus* infection in humans, the staphylococcal antigens are presented to the human immune system in a rather different manner than during the immunization of mice with the purified antigens. In particular, the antigens as presented on the *S. aureus* cell surface might expose different epitopes than when these antigens are presented as soluble recombinant or chemically synthesized proteins in the context of an adjuvant. On the other hand, the eight antigens used for the immunization of mice are also released into the extracellular environment of *S. aureus*, so this cannot be the only explanation for the observed lack of protection upon immunization. Another conceivable explanation is that antibodies against staphylococcal antigens provide less protection against infections than is often assumed. However, the finding that monoclonal antibodies against *S. aureus* antigens, IsaA in particular, as described by the team of Knut Ohlsen at the University of Würzburg and as described in Chapter 5 of this thesis, argues against a lack of protective effects of anti-staphylococcal antibodies. As to the antigens themselves, as they were used in the immunization study described in
Chapter 4, a few additional possibilities remain why they failed to elicit a protective response. Firstly, one or more of them might somehow suppress the murine immune system. This could for example be the case for the four PSMα’s included in the octa-valent antigen mix, since they are known to be toxic for immune cells. Secondly, although the antigens produced in *L. lactis* appear to be correctly folded, they may differ in detail from the authentic antigens produced by *S. aureus*. For example, IsaA, pro-Atl, LytM and Nuc were produced with a C-terminal His$_6$-tag, which would alter the C-terminus of these antigens. This could then lead to an aberrant or at least ineffective epitope recognition by the murine immune system. Evidence for this idea might be deduced from the studies presented in Chapter 6 of this thesis as discussed below. Lastly, as shown in Chapter 3, there may be differences in post-translational modifications, such as phosphorylation, of particular antigens produced by the native host *S. aureus* or the heterologous expression host *L. lactis*. Future vaccination studies should definitely take these different options into consideration and, in particular, the removal of the tags used for antigen purification should be considered. In fact, with this in mind, a vector encoding a tobacco etch virus protease cleavage site allowing removal of the N-terminal His$_6$-tag has already been developed (Chapter 3).

Based on literature and proteome studies the peptidoglycan hydrolase IsaA was identified as a candidate antigen to be targeted in a passive immunization approach with a fully human monoclonal antibody (humAb). In particular, a previous study by the group of Ohlsen had reported the isolation of a murine mAb targeting IsaA. This murine mAb as well as a humanized derivative were shown to be protective in mouse models of catheter-related *S. aureus* infection and *S. aureus* sepsis. In Chapter 5 the identification and production of the fully human anti-IsaA IgG1 humAb 1D9 is described. This humAb was shown to bind to both purified IsaA and native IsaA expressed by 26 different clinical *S. aureus* isolates, including MSSA and MRSA strains. Importantly, in a murine bacteremia model, a significantly improved survival (25-42%) of mice challenged with the clinical *S. aureus* isolate P (MSSA) was observed when humAb 1D9 was provided prophylactically. When mice were challenged with the notorious MRSA strain USA300, 17-42% improved survival of the mice was observed but, unfortunately, this difference was not statistically significant.
Also, no enhanced survival was observed when 1D9 was applied therapeutically upon infection with either \textit{S. aureus} isolate P or USA300. Different possible explanations can be entertained for the apparently limited protection provided by passive immunization with humAb 1D9. One aspect to be considered in the prophylactic application of 1D9 is that \textit{S. aureus} USA300 produced much higher amounts of the IgG-binding protein A than the \textit{S. aureus} isolate P, which may have resulted in the evasion of 1D9-mediated protection by the USA300 strain due to the capture of this antibody by its Fc domain. As the IsaA amino acid sequence is highly conserved among \textit{S. aureus} strains, strain-specific differences in the epitope structure of the IsaA proteins of USA300 and isolate P are a less likely explanation, but in absence of the genome sequence of isolate P this possibility cannot be excluded as yet. A possible explanation for the lack of effect of 1D9 in the investigated therapeutic setting could be an insufficient bioavailability of this humAb. At the time point at which 1D9 was administered to the mice, both \textit{S. aureus} isolate P and USA300 had already multiplied and disseminated to different organs, potentially allowing the bacterial cells to escape from 1D9-mediated elimination. At the present stage, it is only possible to speculate about the reasons why 1D9 did not provide protection in all settings tested. Clearly, further experiments with variations in the dosage and timing of 1D9 administration will be needed to clarify its full potential for prophylactic or therapeutic applications in the protection of patients against \textit{S. aureus} infections. Yet, the fact that this humAb does provide protection in at least one setting suggests that it is in principle possible to apply 1D9 for antistaphylococcal therapy, if only in combination with a classical antibiotic or other specific monoclonal antibodies directed against invariant antigens of \textit{S. aureus}.

The active and passive immunization approaches described in \textbf{chapters 4 and 5} are in line with published studies indicating that, in principle, it should be possible to use such immunization approaches to protect against or cure \textit{S. aureus} infections. In this context, IsaA has presented itself as a particularly promising target, because it is the most immunogenic \textit{S. aureus} protein in humans that we know today, and because monoclonal antibodies against IsaA were found to be at least partially protective against \textit{S. aureus} in murine infection models. Yet, active immunization of mice with IsaA did not elicit
protective antibody responses. The latter observation was puzzling, and it suggested that there might perhaps be differences in the recognition of particular IsaA epitopes that could either lead to the generation of protective or non-protective antibodies. This idea was investigated in the studies described in chapter 6 of this thesis, which showed that there are indeed important differences in the recognition of IsaA by potentially protective and non-protective antibodies. In particular, the partially protective humAb 1D9 was found to bind to an epitope in the N-region of IsaA which was also recognized by presumably protective anti-IsaA antibodies of patients with the genetic blistering disease EB. In contrast, the non-protective antibodies in sera from mice immunized with IsaA were shown to bind predominantly to the C-terminal region of IsaA. Together, these observations seem to suggest that the N-terminal region of IsaA should be targeted in active or passive immunization approaches in order to achieve protection against S. aureus infections.

In conclusion, the present PhD research describes a pipeline that can be applied for the development of potential active or passive immunization approaches to protect frail individuals against infections with S. aureus. Compared to the originally conceived pipeline as presented in Chapter 1 of this thesis, several possible routes have become less attractive, while two converging lines of experimentation have emerged as the most promising options. This is schematically represented in Figure 1. For the development of both passive and active immunization approaches, it is probably best to start with blood donations from patients whose immune system has been heavily challenged by S. aureus on the one end, and with a proteomics analysis for the identification of invariantly expressed S. aureus epitopes on the other end. These lines converge in the mapping of epitopes recognized by potentially protective antibodies in the donated blood samples. As exemplified in chapter 6, such an epitope mapping analysis will require the recombinant production of the respective antigens, or fragments thereof, and the toolbox described in chapters 2 and 3 can be implemented for this purpose. For active immunization approaches, the recombinantly produced antigens can then directly be applied for testing in animal studies as exemplified in chapter 4, whereas the same antigens can also be used for the selection of the humAbs that are to be used for passive immunization approaches as exemplified in chapter 5. Notably, at
the present stage this approach is still somewhat hypothetical, but the good news is that it can now be tested in a relatively straightforward manner.

Figure 1. Schematic representation of the simplified pipeline for development and testing of passive and active immunization strategies against *S. aureus* infections.

**A. Route for the development of an active immunization strategy.** Identification of invariant surface-exposed antigens from *S. aureus* isolates from healthy carriers or infected patients can be achieved by proteomics. Subsequently, several of such antigens or fragments thereof may be produced in a *L. lactis*-based expression system for the mapping of epitopes that are recognized by potentially protective antibodies from donors who have been heavily challenged by *S. aureus* colonization or infection. Lastly, the most appropriate purified recombinant antigens need to be tested, individually or in combination, for their potential applicability in active immunization against *S. aureus* infection using different mouse models.

**B. Route for the development of a passive immunization strategy.** B-cells producing potentially protective antibodies need to be isolated from blood donations from individuals who have been ‘naturally’ challenged by *S. aureus* colonization or infection. Subsequently, the isolated B-cells need to be screened for production of IgGs binding to antigens identified through the epitope mapping analysis described under A. The respective IgGs then need to be cloned and tested for protection against *S. aureus* infection in appropriate animal models.