Article details

Dalen R. van, Molendijk M.M., Ali S., Kessel K.P.M. van, Aerts P., Strijp J.A.G. van, Haas C.J.C. de, Codée J.D.C & Sorge N.M. van (2019), Do not discard Staphylococcus aureus WTA as a vaccine antigen, Nature 572(7767): E1-E2.
Doi: 10.1038/s41586-019-1416-8

MATTERS ARISING

https://doi.org/10.1038/s41586-019-1416-8

Do not discard *Staphylococcus aureus* WTA as a vaccine antigen

Rob van Dalen[1], Michèle M. Molendijk[1], Sara Ali[2], Kok P. M. van Kessel[1], Piet Aerts[1], Jos A. G. van Strijp[1], Carla J. C. de Haas[1], Jeroen Codée[2] & Nina M. van Sorge[1]*

ARISING FROM D. Gerlach et al. *Nature* https://doi.org/10.1038/s41586-018-0730-x (2018)

Wall teichoic acid (WTA) represents a promising vaccine target against antibiotic-resistant *Staphylococcus aureus* (MRSA). In a previous study, Gerlach et al.[1] identified an alternative glycan modification on WTA, which is shown to be poorly immunogenic and helps to subvert antibody recognition, and conclude that vaccines that are directed against glycosylated WTA may fail against many pandemic MRSA clones. However, here we show that this alternative WTA epitope actually represents a highly dominant antigen in the reactive antibody pool against *S. aureus* in humans. In addition, another research group demonstrated that WTA–protein conjugates had strong immunogenicity in mice[2]. Therefore, we believe that WTA should not be discarded as an antigen for vaccines that target *S. aureus* and urge scientists from academic institutions as well as pharmaceutical industry to continue their efforts in this area.

Gerlach et al.[1] found that the genomes of a considerable proportion of the human disease- and livestock-associated MRSA strains contain a prophage that encodes the alternative WTA glycosyltransferase TarP, which modifies WTA with β-1,3-*N*-acetylglucosamine (GlcNAc)[1]. This modification is different from the reactions catalysed by the previously identified glycosyltransferases TarM and TarS, which modify WTA with α-1,4-GlcNAc and β-1,4-GlcNAc, respectively[3,4]. To assess whether human antibodies are able to discriminate between β-1,3-GlcNAc WTA and β-1,4-GlcNAc WTA, Gerlach et al.[1] compared IgG deposition on wild-type and mutant *S. aureus* strains that lacked TarP and/or TarS, using different IgG preparations. In all preparations, more IgG was deposited on bacteria that lacked TarP—that is, those that lacked β-1,3-GlcNAc on WTA. From these data, they concluded that only a small percentage of *S. aureus*-directed antibodies bind to β-1,3-GlcNAc WTA. However, one of the IgG pools used by Gerlach et al.[1] contained affinity-purified IgG against WTA isolated from *S. aureus* strain RN4220. As this strain expresses TarM and TarS but not TarP, this enrichment skewed the antibody pool towards detection of β-1,4-GlcNAc WTA and in fact depleted antibodies specific to β-1,3-GlcNAc WTA. In addition, the sensitivity of the used assay is highly limited (even though *spa* was deleted in the test strains), as all other IgG preparations that Gerlach et al.[1] used contained a diverse anti-*S. aureus* IgG repertoire in addition to WTA-specific IgG, which results in considerable background binding of IgG. Therefore, we believe that the bacterial model that Gerlach et al.[1] used to measure antibody deposition is not sufficiently sensitive and is in part biased towards their conclusion.

We have recently developed a model to study interactions between antibodies and *S. aureus* WTA using chemically defined biotinylated ribitol-phosphate (RboP) hexamers. These molecules are enzymatically modified by TarS, TarM or TarP, yielding β-1,4-GlcNAc WTA (TarS–WTA), α-1,4-GlcNAc WTA (TarM–WTA) and β-1,3-GlcNAc WTA (TarP–WTA). This model circumvents the sensitivity limitations that are associated with the use of whole bacteria and precludes the need for IgG affinity purification, enabling the specific and sensitive detection of WTA-interacting antibodies in whole serum. To validate our model, we tested the deposition of previously characterized human monoclonal IgG1 antibodies against either β-1,4-GlcNAc (clone 4497) or α-1,4-GlcNAc (clone 4461) on synthetic WTA that was immobilized on beads[5]. Both monoclonal IgG1 antibodies bound to their respective ligands with complete exclusiveness (Fig. 1a). Notably, the β-1,4-GlcNAc monoclonal IgG1 antibody could not distinguish between TarS–WTA and TarP–WTA, indicating that anti-β-GlcNAc antibodies can be cross-reactive to TarS–WTA and TarP–WTA. To ensure that this was not a unique property of monoclonal antibody 4497, we expressed two additional anti-β-1,4-GlcNAc IgG1 monoclonal antibodies (clones 4462 and 6292)[5]. Again, both monoclonal antibodies recognized both TarS–WTA and TarP–WTA, although monoclonal antibody 6292 showed some level of discrimination (Fig. 1a).

To repeat the previously published[1] IgG-deposition experiment, we measured deposition of IgG using pooled human serum or the isolated total IgG fraction from this pool on TarP–WTA, TarS–WTA, TarM–WTA or unglycosylated RboP (Fig. 1b). Consistent with data obtained by others[6,7], anti-TarS–WTA IgG levels were approximately tenfold higher than anti-TarM–WTA IgG in serum, whereas RboP-specific IgG was undetectable. Notably, the difference between anti-TarS–WTA IgG and anti-TarP–WTA IgG levels was only twofold (Fig. 1b). Thus, the β-1,3-GlcNAc WTA epitope seems to be the second-most dominant *S. aureus* antigen, after β-1,4-GlcNAc WTA, which is reportedly targeted by 70% of all *S. aureus*-reactive IgG[8]. This is in contrast to the suggestion by Gerlach et al.[1] that only a small percentage of *S. aureus*-specific IgG antibodies target this epitope. We next repeated the phagocytosis experiment using our low-background WTA bead model. Notably, our set-up allowed the use of full serum, thus preventing bias that could be introduced by affinity purification. Phagocytosis of WTA beads was proportional to the level of TarS- and TarP-reactive antibodies; phagocytosis of TarP–WTA beads was approximately half of the phagocytosis levels of TarS–WTA beads (Fig. 1c). This confirmed that TarP–WTA-reactive antibodies were fully functional. However, it is currently unknown what the levels of phagocytosis are that are required for the protection against *S. aureus*, making it difficult to interpret this twofold difference with regard to biological relevance.

We also assessed inter-individual differences in antibody reactivity against TarP–WTA and TarS–WTA using sera from 11 individual donors, whose serum was included in the serum pool. The levels of anti-TarS–WTA and anti-TarP–WTA IgG2, the predominant IgG subclass that targets *S. aureus* WTA[9], were highly variable per donor (Fig. 1d). Notably, in 5 out of 11 donors, IgG2 interacted to a similar extend against TarP–WTA and TarS–WTA. Furthermore, these levels were highly correlated ($R^2 = 0.74$; Fig. 1d), indicating that these two antibody

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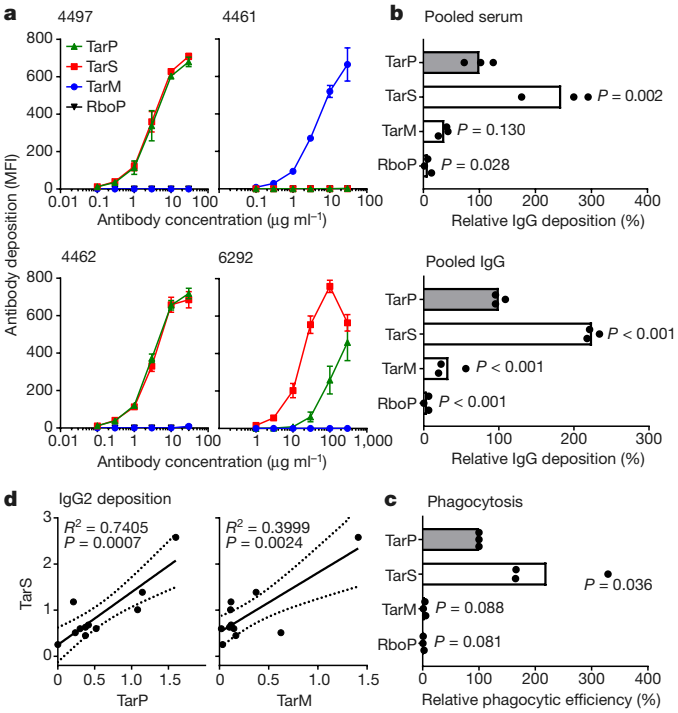

Fig. 1 | TarP-modified WTA is recognized by human IgG antibodies that induce phagocytosis. a, Deposition of monoclonal IgG1 antibodies specific to β-1,4-GlcNAc (clones 4497, 4462 and 6292) or α-1,4-GlcNAc (clone 4461) WTA on beads coated with TarP–WTA, TarS–WTA, TarM–WTA or unglycosylated RboP. The fluorescence intensity (geometric mean ± s.d. of three independent experiments) is shown. MFI, mean fluorescence intensity. **b**, IgG deposition on beads coated with TarP–WTA, TarS–WTA, TarM–WTA or RboP using 3% pooled human serum or pooled human IgG at 10 μg ml^{-1}. Data were normalized to the mean TarP–WTA values. Bars represent the mean of three independent experiments, and each dot indicates the data point from an individual experiment. Data were corrected for background based on binding to biotin control beads. Significance tested by one-way analysis of variance (ANOVA) followed by Dunnett's test. **c**, Neutrophil phagocytosis of FITC (fluorescein isothiocyanate)-labelled beads coated with TarP–WTA, TarS–WTA, TarM–WTA or unglycosylated RboP opsonized with 1% pooled human serum. The relative phagocytic efficiency was assessed as the geometric mean fluorescence intensity, normalized to TarP–WTA of each donor. Significance tested by one-way ANOVA followed by Dunnett's test. Bars represent mean values of three independent experiments using neutrophils from different donors are shown, and each dot indicates the data point from an individual experiment. **d**, Correlation between levels of IgG2 targeting TarS–WTA and TarP–WTA or TarM-WTA in 3% serum of 11 individual donors. Data are mean absorbance at 450 nm of three independent experiments and were analysed by linear regression. Dashed lines indicate the 95% confidence intervals.

pools are potentially cross-reactive against the two β-GlcNAc epitopes. By contrast, the correlation between TarS and TarM IgG2 levels was low ($R^2 = 0.40$; Fig. 1d), suggesting that there is limited cross-reactivity between these antibody pools. The observation of potential cross-reactivity is in line with results from our observations using monoclonal IgG1 antibodies (Fig. 1a) and also with results from others[2] who have demonstrated that antisera raised against TarS–WTA reacted strongly against TarP–WTA. The existence of cross-reactive antibodies between TarS–WTA and TarP–WTA was not described by Gerlach et al.[1].

Finally, the previously performed immunization experiments[1] did not use protein-conjugated WTA, which is a common method to enhance the immunogenicity of carbohydrate antigens[2]. Correspondingly, it has been shown that unconjugated WTA molecules hardly induce IgG production[2], which is in line with the low immunogenicity of β-1,4-GlcNAc WTA that was found in the previously published experiment[1]. Notably, it was also shown that protein-conjugated β-1,3-GlcNAc WTA is highly immunogenic and induces strong IgG production[2]. Therefore, the conclusion of Gerlach et al.[1] that β-1,3-GlcNAc represents a poor vaccine target seems unjustified.

In summary, our data show that serum contains a substantial pool of IgG antibodies that interact with TarP–WTA, thereby demonstrating immunogenicity of this epitope in humans. Similarly, other groups have shown that β-1,3-GlcNAc WTA is immunogenic in mice when WTA is presented as a protein-conjugated vaccine. In contrast to Gerlach et al.[1], we believe that these data demonstrate that WTA modified with β-1,4-GlcNAc or β-1,3-GlcNAc represents a promising vaccine antigen, for which we urge additional studies by academic groups as well as industry. Synthetic WTA oligomers represent valuable tools for the dissection of the immune interaction of *S. aureus* WTA with host molecules, as well as potential vaccine targets.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The raw data associated with Fig. 1 are available from the corresponding author upon request.

Received: 21 December 2018; Accepted: 29 May 2019;
Published online 31 July 2019.

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Acknowledgements This work was supported by a Vidi grant (91713303) from the Netherlands Organization of Scientific Research (NWO) to N.M.v.S. and R.v.D.

Author contributions R.v.D. and N.M.v.S. designed experiments and wrote the manuscript. R.v.D. and M.M.M. performed experiments and prepared figures. K.P.M.v.K., P.A. and C.J.C.d.H. provided technical assistance. S.A. and J.C. produced and provided essential reagents. K.P.M.v.K., J.A.G.v.S. and J.C. provided critical feedback.

Competing interests The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1416-8.
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Last updated by author(s): Mar 29, 2019

Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Flow cytometry data was acquired using FACSuite (BD).
Data analysis	Flow cytometry data were analyzed using FlowJo 10 (FlowJo, LLC). All data was statistically tested using Graphpad Prism 7.02 (GraphPad Software).

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Life sciences study design

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Sample size	As this analysis was descriptive and explorative in nature, no sample size calculations were performed. - Analysis of specific IgG levels was performed using serum or IgG from a pool of 21 donors, and can therefore be considered an average of the respective specific IgG levels of 22 individuals. The experiment was repeated in triplo to allow for statistical analysis by ANOVA. - Interindividual variation of specific IgG2 levels was assessed in 11 donors and was intended as initial exploration of interindividual variation. Combination of data from these donors allowed for sufficient datapoints to perform linear regression analysis. - PMN phagocytosis of beads opsonized with pooled serum from 21 donors was performed using PMNs from 3 unrelated donors to allow assessment of the biological variation between PMN donors and allow for statistical analysis by ANOVA.
Data exclusions	No data were excluded
Replication	All experiments were performed in triplicates and fully replicated in all instances. - Opsonisation was replicated using pooled human serum and isolated IgG from this pool, which yielded the same results. - Phagocytosis was replicated using PMNs from three donors, which yielded the same results.
Randomization	Group allocation was not relevant to this study, as all samples/strains were subjected to all experimental procedures.
Blinding	Blinding was not relevant to this study, as the readouts of all experiments could be assessed objectively (no clinical scores or microscopic analysis).

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	- The following anti-WTA human IgG1 antibodies clones were expressed: 4461, 4497, 4462 and 6292. All were previously characterized, validated and patented by Genentech: WO 2014/193722 A1. - Anti-human-IgG2-HRP was acquired from Invitrogen (Clone HP6014, Cat no 05-0520, Lot no 30212905, used per manufacturer's instructions at 1:500 dilution)
Validation	- All human anti-WTA IgG1 antibodies clones were previously characterized, validated and patented by Genentech: WO 2014/193722 A1. Clone 4461 is reactive with S. aureus WTA alpha-GlcNAc, clones 4497, 4462 and 6292 are reactive with S. aureus WTA beta-GlcNAc. Target specificity of the re-expressed antibodies was verified by assessment of deposition on wildtype as well as target-deficient bacteria (dTarM for clone 4461, dTarS for clones 4497, 4462 and 6292) in the same manner as performed by Genentech. Clones 4461 and 4497 have been characterized further in 10.1038/nature16057. Clones 4462 and 4497 have been characterized further in doi:10.1080/19420862.2018.1501252. - Anti-human-IgG2-HRP secondary antibody is reactive with human Fd, and has been used in several publications (doi:10.1016/S0264-410X(01)00253-5 and PMID: 9660461).

Human research participants

Policy information about studies involving human research participants

Population characteristics	All donors remained anonymous to the researchers, no characteristics are known nor stored.
Recruitment	Participants were recruited from a donor pool and selected based on availability by people unknown to the researchers.
Ethics oversight	Medical ethics committee of the University Medical Center Utrecht (METC-protocol 07–125/C approved on March 1, 2010)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

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Methodology

Sample preparation	Neutrophils were isolated by Ficoll/Histopaque gradient centrifugation and washed in RPMI + 0.05% HSA. Post-phagocytosis, neutrophils were washed in RPMI + 0.05% HSA and fixed in 1% formaldehyde. Dynabeads M280 Streptavidin coated with glycosylated biotinylated WTA molecules were stained with monoclonal or serum antibodies, which were detected using Protein G-Alexa Fluor 488.
Instrument	BD FACSVerse, Cat. no. 651155
Software	Flow cytometry data was acquired using FACSuite (BD) and analyzed using FlowJo 10 (FlowJo, LLC).
Cell population abundance	No fluorescence activated cell sorting was performed.
Gating strategy	Neutrophils were gated on SSC/FSC, using a tight FSC threshold and a wide SSC threshold, to allow for detection of neutrophils that have engulfed one or multiple beads. Dynabeads were gated using a tight SSC/FSC gate, based on unstained samples. No discrimination between positive/negative populations was made.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

https://doi.org/10.1038/s41586-019-1417-7

Reply to: Do not discard *Staphylococcus aureus* WTA as a vaccine antigen

David Gerlach[1,2], Yinglan Guo[3], Thilo Stehle[3] & Andreas Peschel[1,2]*

REPLYING TO R. van Dalen et al. *Nature* https://doi.org/10.1038/s41586-019-1416-8 (2019)

Recently, we described[1] that the shift of an *N*-acetylglucosamine (GlcNAc) group from the C4 to the C3 atom of the ribitol-phosphate (RboP)-repeating unit of the wall teichoic acid (WTA) of *Staphylococcus aureus* strongly reduced the capacity of mice to mount a protective IgG response. This finding was consistent with the low levels of IgG directed against the altered WTA that were found in human sera[1]. The unusual glycosylation pattern of WTA was introduced by the enzyme TarP, which is expressed by prophages that are found in many major methicillin-resistant *S. aureus* lineages and we found that this unusual glycosylation pattern of WTA increases the capacity of this pathogen to evade recognition by the adaptive immune system. In the accompanying Comment[2], van Dalen et al. provide new experimental data, highlight a recent patent application that uses TarP-modified, protein-conjugated WTA as a vaccine antigen against *S. aureus*[3] and conclude that TarP-modified WTA (TarP–WTA), conjugated to a suitable carrier protein, should remain on the list of promising vaccine candidates.

Glycopolymers—such as lipopolysaccharides (in Gram-negative bacteria), teichoic acids (in Gram-positive bacteria) or capsular polysaccharides—dominate the molecular composition of bacterial surfaces and are promising antigens for protective immune responses, because glycopolymers are composed of highly repetitive and largely invariant glycoepitopes[4] that are often species or strain-specific. However, glycopolymers are generally difficult to target by adaptive immune responses, because only under certain circumstances can antigen-presenting cells effectively present such polymers and this has important consequences for the responses of B and T cells. Glycopolymers are potent antigens when covalently conjugated to carrier proteins that facilitate their presentation, for example, in vaccines directed against *Haemophilus influenceae*, *Neisseria meningitidis*, *Streptococcus pneumoniae* or *Salmonella typhi*[5]. Furthermore, some bacterial glycopolymers can be presented in major histocompatibility complex (MHC) class II molecules and stimulate lymphocytes without conjugation to carrier proteins. These include the capsular polysaccharides of *Bacteroides fragilis*, *S. aureus* and *S. pneumoniae*[6], and WTA of *S. aureus*[7,8]. However, the capacity to present native, unconjugated glycopolymers depends on certain structural features of these molecules that include zwitterionic properties[6]. The exact structural requirements and molecular mechanisms that are necessary for MHC class II presentation remain poorly understood.

We appreciate the contribution of van Dalen et al.[2] and agree with most of the points made in the Comment. The described experimental set-up is based on IgG that binds either to beads or to enzyme-linked immunosorbent assay (ELISA) plates that are coated with semi-synthetic WTA. The study demonstrates that TarP–WTA is bound by IgG from human sera at a much lower level than TarS–WTA. The observed differences are in fact similar to those found in our previously published paper using whole bacterial cells[1], thereby supporting our findings. The assay system of van Dalen et al.[2] enables the quantification of IgG–WTA binding in the absence of other *S. aureus* antigens, with a much lower amount of background binding. However, we do not fully agree with all of the conclusions that were drawn by van Dalen et al.[2].

First, van Dalen et al.[2] conclude based on the fact that TarP–WTA binds to much lower, but still reasonable, amounts of human serum IgG that TarP–WTA must be immunogenic in humans, which is in contrast to our previous findings[1] that native, unconjugated TarP–WTA was not or only weakly immunogenic in mice. It should be noted that van Dalen et al.[2] showed that several monoclonal antibodies directed against WTA, modified by the housekeeping glycosyltransferase TarS[9] (TarS–WTA; Fig. 1), cross-reacted with TarP-WTA. Thus, the comparatively low level of TarP–WTA binding by antibodies from human serum could be due to limited cross-reactivity of human antibodies, which use TarS–WTA as their major antigen, and it does not necessarily mean that native TarP–WTA is immunogenic in humans.

Second, van Dalen et al.[2] suggest that the selection of our human serum samples may have biased the results, because we used, in one of the IgG-binding experiments, human IgG that was enriched for binding to WTA from an *S. aureus* strain that lacked *tarP*. We would like to emphasize that we also showed five other non-enriched human serum preparations, which were pooled or from individual donors, all of which consistently showed the characteristic difference in IgG binding to TarP–WTA compared to TarS–WTA, albeit with the expected individual variation[1]. As outlined above, the relative differences in binding of human serum IgG to TarP–WTA and TarS–WTA are quite similar in both the study described by van Dalen et al.[2] and our previous study[1].

van Dalen et al.[2] also highlighted the recent patent filed by Driguez and colleagues[3], in which the authors analysed the immunogenic potential of unconjugated and conjugated WTA variants. The study described in the patent[3] reported that after conjugation with a carrier protein, a robust IgG response was elicited by vaccination of mice with synthetic TarP–WTA or TarS–WTA oligomers. By contrast, neither of the two types of WTA provoked an IgG response as unconjugated molecules, which partly contradicts the results of our previous study, in which we show that native TarS–WTA, but not TarP–WTA, has strong immunogenicity[1]. It should be noted that the unconjugated TarS–WTA and TarP–WTA preparations used in the study described in the patent were devoid of D-alanine residues, which introduce positive charges into the otherwise negatively charged repeating units of WTA[10]. Accordingly, ^{1}H-NMR data of the isolated WTA showed no presence of D-alanine residues[3]. Consequently, the WTA molecules were not zwitterionic and had probably lost the capacity to be presented by MHC class II molecules and to activate lymphocytes. By contrast, our WTA preparations had maintained the D-alanine esters as confirmed by NMR analysis[1], which may have been the reason for the observed immunogenicity of unconjugated TarS–WTA.

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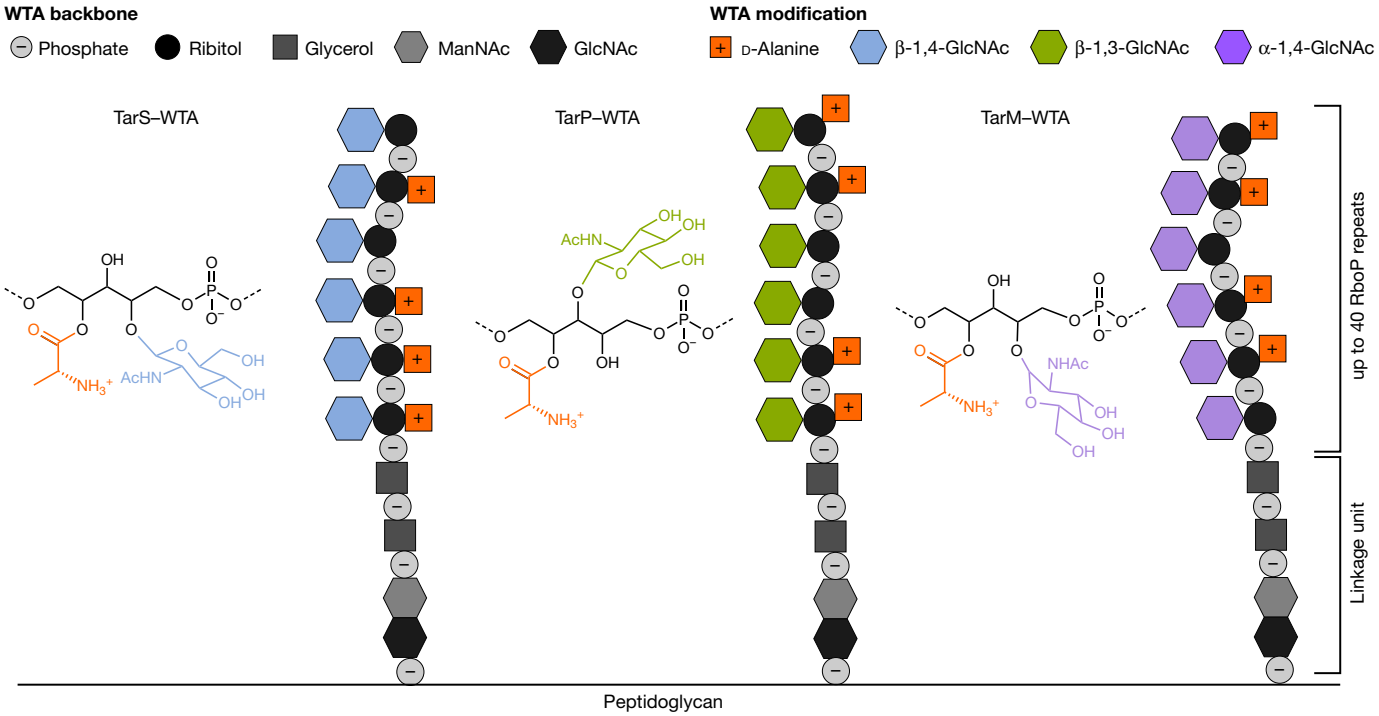

Fig. 1 | Structure of *S. aureus* WTA and its variation by different GlcNAc transferases. The three identified RboP WTA variants generated by the glycosyltransferases TarS, TarP and TarM are shown. ManNAc, *N*-acetylmannosamine.

Lastly, we are excited about the findings of van Dalen et al.[2] using *S. aureus* WTA modified by the alternative glycosyltransferase TarM, which modifies RboP-repeating units with GlcNAc in the α configuration[11] (rather than TarS or TarP, which mediated modifications in the β configuration[1] (Fig. 1)). TarM–WTA showed even weaker binding by human serum antibodies than TarP–WTA, suggesting that TarM, which is found in a number of clonal lineages of *S. aureus*, may have an even stronger influence on the immune evasion abilities of *S. aureus* than TarP.

In conclusion, the findings described by van Dalen et al.[2] and in our previous study[1] are highly congruent, although using different serum preparations and different experimental strategies. The focus on results from the recent patent of Driguez et al.[3] demonstrates that even TarP–WTA can be a potent antigen for a protective vaccine when conjugated to a suitable carrier protein. We therefore agree that glycosylated WTA should not be discarded as a vaccine antigen, because it is highly abundant at the bacterial surface and could be a suitable target for opsonic antibodies. The discovery of TarP and its influence on immune recognition adds another important pathogenicity factor to the virulence factor arsenal of *S. aureus* and it underscores the importance of adaptive immunity and the evasion of the adaptive immune system by *S. aureus* during infections with this pathogen.

D.G., Y.G., T.S. and A.P. contributed to drafting and reviewing the data, and wrote the Reply. The other authors of the original study[1] were not involved in the preparation of this Reply.

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Acknowledgements Our work was financed by grants from the German Research Foundation to A.P. (TRR34, CRC766, TRR156 and RTG1708) and T.S. (TRR34, CRC766) and the German Center for Infection Research and is supported by infrastructure provided by the Cluster of Excellence (EXC 2124).

Author contributions D.G, Y.G., T.S. and A.P analysed data and wrote the manuscript.

Competing interests The authors declare no competing interests.

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