Peptidoglycan Contribution to the B Cell Superantigen Activity of Staphylococcal Protein A

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ABSTRACT Staphylococcus aureus causes reiterative and chronic persistent infections. This can be explained by the formidable ability of this pathogen to escape immune surveillance mechanisms. Cells of S. aureus display the abundant staphylococcal protein A (SpA). SpA binds to immunoglobulin (Ig) molecules and coats the bacterial surface to prevent phagocytic uptake. SpA also binds and cross-links variable heavy 3 (VH3) idiotype (IgM) B cell receptors, promoting B cell expansion and the secretion of nonspecific V\textsubscript{H}3-IgM via a mechanism requiring CD4\textsuperscript{T} T cell help. SpA binding to antibodies is mediated by the N-terminal Ig-binding domains (IgBDs). The so-called region X, uncharacterized LysM domain, and C-terminal LPXTG sorting signal for peptidoglycan attachment complete the linear structure of the protein. Here, we report that both the LysM domain and the LPXTG motif sorting signal are required for the B cell superantigen activity of SpA in a mouse model of infection. SpA molecules purified from staphylococcal cultures are sufficient to exert B cell superantigen activity and promote immunoglobulin secretion as long as they carry intact LysM and LPXTG motif domains with bound peptidoglycan fragments. The LysM domain binds the glycan chains of peptidoglycan fragments, whereas the LPXTG motif is covalently linked to wall peptides lacking glycan. These findings emphasize the complexity of SpA interactions with B cell receptors.

IMPORTANCE The LysM domain is found in all kingdoms of life. While their function in mammals is not known, LysM domains of bacteria and their phage parasites are associated with enzymes that cleave or remodel peptidoglycan. Plants recognize microbe-associated molecular patterns such as chitin via receptors endowed with LysM-containing ectodomains. In plants, such receptors play equally important roles in defense and symbiosis signaling. SpA of S. aureus carries a LysM domain that binds glycan strands of peptidoglycan to influence defined B cell responses that divert pathogen-specific adaptive immune responses.

KEYWORDS B cell receptor, LysM, protein A, Staphylococcus aureus, peptidoglycan, superantigens
(Fab), which is encoded by variable heavy 3 (V\textsubscript{H}3) clan-related genes (13). SpA can cross-link the V\textsubscript{H}3-IgM B cell receptors (BCRs) to trigger supraclonal proliferation of B cells (9, 11). During infection of mice and humans, B cell superantigen activity leads to the expansion of V\textsubscript{H}3 idotype plasmablasts in blood and to the secretion of V\textsubscript{H}3 idio-
type antibodies that lack recognition of staphylococcal antigens (12, 14). In this man-
ner, SpA may contribute to the lifelong association of S. aureus with humans and the bacterium’s ability to cause reiterative infections (15).

SpA is synthesized as a precursor in the bacterial cytoplasm and enters the secre-
tory pathway via an N-terminal signal peptide (16). Once translocated across the plasma membrane, the signal peptide is cleaved by signal peptidase and sortase A (SrtA) cleaves the C-terminal LPXTG sorting signal after threonine (T) and transfers the processed polypeptide to the terminal glycine (G) residue of the peptidoglycan intermediate [C\textsubscript{55}-(PO\textsubscript{4})\textsubscript{2}-MurNAc(L-Ala-d-iGlu-L-Lys(NH\textsubscript{2}-Gly\textsubscript{5})\textsubscript{d}-Ala-o-Ala)-GlcNAC] known as lipid II (17, 18). The SpA-lipid II intermediate is then incorporated into cell wall peptido-
glycan and displayed on the bacterial surface (19). During cell division, staphylococci release a portion of their peptidoglycan and peptidoglycan-linked SpA into the extracell-
ular milieu, owing to muralytic enzymes that degrade the cell wall in the vicinity of septal membranes (20–22). Released SpA is tethered to wall peptide with the C-termi-
nal structure L-Ala-d-iGln-L-Lys(SpA-LPET-Gly\textsubscript{5})-d-Ala-Gly\textsubscript{4} (23). Native SpA released from the staphylococcal cell wall, but not recombinant SpA purified from the cytoplasm of Escherichia coli, is sufficient to induce the expansion of V\textsubscript{H}3 idotype IgG and IgM in mice (12).

In this study, we asked whether SpA modification with peptidoglycan is necessary for the expansion of V\textsubscript{H}3 clonal immunoglobulin. By engineering strains that carry the spa gene lacking defined domains of the protein, we observed that S. aureus spa\textsubscript{DLPXTG}, a variant that can no longer immobilize SpA in the envelope, fails to exhibit B cell superantigen activity in vivo and in vitro. Further, the predicted LysM domain of SpA, positioned between the envelope spanning region X (Xr) and the LPXTG sorting signal, also contributes to B cell superantigen activity by binding the glycan chains of pepti-
doglycan fragments. Thus, together with peptidoglycan linked to the LPXTG motif, the LysM domain activates immune cells to implement the B cell superantigen activity of staphylococcal protein A.

RESULTS

S. aureus spa variants lacking defined C-terminal subdomains of protein A. The translational product of the S. aureus Newman spa gene encompasses an N-terminal signal peptide, five IgBDs of 58 to 61 residues (designated E, D, A, B, and C), the so-
called region X (Xr) with 10 tandem repeats of the octapeptide EDNNKPGK, a predicted LysM domain, and a C-terminal LPXTG sorting signal (Fig. 1A). Earlier work demonstrated that staphylococcal secretion of SpA encompassing four or five IgBDs is essen-
tial for B cell superantigen activity (12). To investigate the contribution of the C-termi-
nal domains of SpA to B cell superantigen activity, we generated S. aureus Newman variants with modi-
fi ed spa genes that lacked coding sequences for either Xr (spa\textsubscript{DXr}), LysM (spa\textsubscript{DLysM}), or the LPXTG sorting signal (spa\textsubscript{DLPXTG}) (Fig. 1A). Production of the new SpA variants was assessed by immunoblotting extracts derived from cultures of bacte-
ria fractionated into supernatant (S), cell wall (W), membrane (M), and cytoplasm (C). The S. aureus Newman \textDelta spa strain, which does not produce SpA, was used as a control. Full-length SpA and SpA\textsubscript{DXr} and SpA\textsubscript{DLysM} lacking the C-terminal Xr and LysM domains, respectively, displayed similar fractionation profiles, with most of the proteins found in the cell wall (Fig. 1B). SpA is released from the bacterial peptidoglycan via the combined activities of the murein hydrolases AtlA, Sle, LytM, and LytN (23). Thus, SpA proteins were also found in the culture supernatant (Fig. 1B). SpA\textsubscript{DLPXTG}, which lacks peptidogly-
can anchoring (LPET-COOH), was predominantly found in the culture supernatant (Fig. 1B). While similar between all mutants, total immune-reactive signals for SpA were reduced compared to those for wild-type bacteria. We attribute this difference to the
reduced stability of proteins following secretion in the culture medium. In agreement with this notion, a representative immunoprecipitation experiment shows similar levels of production of SpA and SpA LysM (see Fig. S1 in the supplemental material). Immunoblotting with antibodies against the membrane protein sortase A (anti-SrtA), secreted coagulase (anti-Coa) and cytoplasmic ribosomal protein L6 (anti-L6) was used to validate the fractionation of cultures (Fig. 1B). To further visualize the surface display of SpA, monoclonal antibody 3F6, which binds to the LysM domain, and BODIPY-vancomycin (green) were used to visualize the surface display of SpA.

**FIG 1** C-terminal domains of staphylococcal protein A (SpA) and their impact on the subcellular localization of SpA variants. (A) Diagram illustrating the structure of the primary SpA translation products with a N-terminal signal peptide (white rectangle) and signal peptidase cleavage site, five immunoglobulin binding domains (IgBDs) designated E, D, A, B, and C, region X (Xr) with 10 repeats of an octapeptide, predicted LysM domain, and C-terminal sorting signal cleaved by sortase between the threonine (T) and the glycine (G) of its LPXTG motif. Variants SpA Xr, SpA LysM, and SpA LPXTG lack amino acid sequences encompassing the Xr, LysM domain, and LPXTG sorting signal as illustrated. rSpA depicts recombinant SpA produced in E. coli without a signal sequence and sorting motif. Molecular weights corresponding to the primary sequence of proteins are shown. (B) Fractionation of S. aureus cultures into culture supernatant (S), lysostaphin-digested cell wall (W), membrane (M), and cytoplasm (C) and immunoblot analysis for the distribution of wild-type and mutant SpA. Antibodies against sortase, coagulase, and ribosomal protein L6 were used to control for successful fractionation. (C) BODIPY-vancomycin (green) staining of peptidoglycan and Alexa Fluor 647-labeled secondary antibody (red) detection of SpA monoclonal antibody 3F6 binding to S. aureus were used to visualize the surface display of SpA.
dependent manner (26). We wondered whether mice infected with *S. aureus* spa

SPA D Xr, spa D LysM, spa D LPXTG, and Δspa variants continue to expand V H3 clonal antibodies. Because the variants produced less SpA protein, we first compared the abilities of the new strains to disseminate and replicate in mouse kidneys following intravenous infection. At day 5 postinfection, only animals infected with the Δspa variant displayed a significant reduction in bacterial loads in kidney tissues (Fig. 2A). Next, cohorts of animals were infected with the same strains; blood was collected on days 5, 12, 19, and 26 postinfection. Serum samples were subjected to enzyme-linked immunosorbent assay (ELISA) with SpAKK, a protein A variant that binds VH3 variant heavy chains but not Ig Fc (12). Compared to mock-infected animals, mice infected with wild-type *S. aureus* exhibited a large expansion of VH3 idiotype IgM in blood on day 5 postinfection (Fig. 2B). VH3 idiotype IgM expansion abated by day 12 and was accompanied by a rise in VH3 idiotype IgG sustained over 26 days (Fig. 2C). As expected, animals infected with the Δspa mutant did not display B cell superantigen activity (Fig. 2B and C). Although slightly reduced compared to the case with the wild type, VH3 antibody expansions were also observed in animals infected with the spa ΔXr variant. Infection of mice with *S. aureus* spa ΔLysM caused a modest expansion of VH3 idiotype IgM on day 5, but unlike the case with the wild-type strain or the spa ΔXr variant, failed to activate VH3 idiotype IgG expansion on day 12 (Fig. 2B and C). Animals infected with the spa ΔLPXTG mutant did not display any B cell superantigen activity, corroborating the notion that peptidoglycan modification of naturally released SpA is key to its B cell superantigen activity (Fig. 2B and C). The data suggest that the LysM domain also contributes to SpA-induced expansions of VH3 idiotype immunoglobulins during infection.

**Human B cell proliferation induced by *S. aureus* spa variants.** Previous work reported that heat-killed *S. aureus* cells or staphylococcal extracts induce *in vitro* proliferation of isolated human B cells in a T cell-independent manner (8, 27). To analyze the contribution of specific domains of SpA in this context, *S. aureus* Newman or its spa ΔXr, spa ΔLysM, spa ΔLPXTG, and Δspa variants were fixed with 0.5% formaldehyde, heat killed, washed in buffer, and added to B cells isolated from human blood mononuclear cell preparations using negative selection (Fig. S2A) or positive selection (Fig. S2B). Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-stained CD19+ cells was quantified by measuring dilution of fluorescence over 6 days (Fig. S2C). Compared to mock-treated lymphocytes, incubation of mixed populations of CD19+ B cells/CD3+ T

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**FIG 2** The LPXTG motif and LysM domain of SpA are required for *S. aureus* B cell superantigen activity in mice. (A) Cohorts of BALB/c mice (*n* = 10) were infected intravenously with 1 × 10^7 CFU of the *S. aureus* Newman wild type (WT) or spa variant strains. Five days postinfection, kidneys were removed and ground for enumeration of CFU per gram of tissue. (B and C) Cohorts of BALB/c mice (*n* = 10) were infected via intravenous injection with 1 × 10^7 CFU of *S. aureus* or a control (PBS [mock]). On days 5, 12, 19, and 26, serum was collected via retro-orbital bleeding and IgM (B) and IgG (C) responses were quantified by ELISA using SpAPo, a SpA variant that cannot bind IgG Fc but retains the ability to bind VH3 variant heavy chains of IgG and IgM. Statistical analysis was performed with one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test. **,** *P* < 0.01; *P* < 0.05. ns, not significant.
cells or purified CD19⁺ B cells with formalin-fixed *S. aureus* Newman at a ratio of 16 bacteria/cell caused proliferation of 16.93% ± 5.16% and 29.36% ± 2.94% of CD19⁺ B cells, respectively (Fig. 3A and B). In contrast, incubation of B cells with the Δspa mutant did not activate proliferation compared to that with phosphate-buffered saline (PBS) (Fig. 3A and B). Incubation of CD19⁺ B cells/CD3⁺ T cells with *S. aureus* spaΔXr caused proliferation of CD19⁺ B cells in a manner similar to that of wild-type staphylococci (Fig. 3A). However, a small proliferation reduction was observed when *S. aureus* spaΔXr was incubated with purified CD19⁺ B cells (Fig. 3B). This difference may represent a small contribution of the Xr domain or reflect heterogeneity in blood donors. Incubation with formalin-fixed spaΔLPXTG or Δspa bacteria significantly reduced CD19⁺ B cell proliferation (Fig. 3A and B). This is as expected since SpAΔLPXTG is no longer retained in the envelope of bacteria and thus is washed away during the preparation of fixed cells. Interestingly, CD19⁺ B cell proliferation was also significantly reduced upon incubation with spaΔLysM fixed bacteria (Fig. 3A and B). To ascertain that the phenotype was not the result of reduced display of SpAΔlysm, three new strains were generated whereby the wild-type spa, spaΔXr, and spaΔlysm genes were expressed under the control of the tetracycline-inducible promoter. The new strains, the itet-spa, itet-spaΔXr, and itet-spaΔlysm variants, were grown to similar densities with adjusted concentrations of inducer to produce similar amounts of SpA proteins (Fig. 4A). Incubation of CD19⁺ B cells/CD3⁺ T cells with the itet-spaΔlysm strain resulted in reduced expansion of B cells compared to that with itet-spa (isogenic wild-type) *S. aureus* (Fig. 4B), confirming that the LysM domain contributes to SpA activity.

As observed with animal infection with live bacteria, the cell expansion of human lymphocytes upon incubation of fixed *S. aureus* Newman or its spaΔXr variant resulted in robust IgM secretion; this was not observed with the spaΔLPXTG and Δspa variants (Fig. 3C). *S. aureus* activation of IgM secretion by CD19⁺ B cells did not require CD3⁺ T
cells, as similar amounts of immunoglobulin secretion were observed when S. aureus Newman or its spa$_{ΔX}$ variant were incubated with purified CD19$^+$ B cells (Fig. 3CD). Thus, whole-cell S. aureus preparations activate CD19$^+$ B cell proliferation in the presence and in the absence of CD3$^+$ T cells. CD19$^+$ B cell expansions are dependent on SpA surface display, as neither Δspa nor spa$_{ΔLPXTG}$ mutant staphylococci could activate proliferation.

**Human B cell proliferation induced by purified SpA variants.** To determine whether SpA alone is sufficient to induce proliferation of human CD19$^+$ B cell populations, full-length SpA and variants Spa$_{ΔX}$, Spa$_{ΔLYM}$, and Spa$_{ΔLPXTG}$ were purified from the filtered medium of S. aureus cultures via affinity chromatography on IgG Sepharose (Fig. 5A). Recombinant SpA (rSpA) (Fig. 1A) starting with six histidyl residues instead of the signal sequence and ending with the sorting motif followed by the Strep-tag sequence (LPETGWSHPQFEK) was purified from the cytosol of E. coli (Fig. 5A). Incubation of mixed populations of CD19$^+$ B cells and CD3$^+$ T cells with SpA caused proliferation of CD19$^+$ B cells (32.46% ± 8.41%), while rSpA did not trigger B cell expansion (Fig. 5B). As a control, subjecting filtered, conditioned medium of centrifuged S. aureus Δspa cultures to affinity chromatography generated a sample that induced proliferation of 9.80% ± 5.37% of CD19$^+$ B cells in the mixed CD19$^+$ B cell/CD3$^+$ T cell populations (Fig. 5B, Δspa mutant versus rSpA, $P=0.01$). We surmise that the mitogenic activity associated with the Δspa sample may be due to lipoprotein or nucleic acid contamination and activation of Toll-like receptors (TLRs) (27). Incubation of CD19$^+$ B cell/CD3$^+$ T cell populations with nonmethylated CpG, a potent inducer of TLR9 signaling (27), induced the proliferation of 29.04% ± 5.68% CD19$^+$ B cells (Fig. 5B). Spa$_{ΔX}$ induced CD19$^+$ B cell proliferation similar to that of SpA, while removal of the LysM domain (SpA$_{ΔLYM}$) and LPXTG sorting signal (SpA$_{ΔLPXTG}$) led to a gradually reduced ability to promote B cell proliferation (Fig. 5B). In fact, Spa$_{ΔLPXTG}$ activation was similar to that observed with Δspa (Fig. 5B). Purified SpA molecules were also added to purified (97 to 99% pure) CD19$^+$ B cells (Fig. 5C). Compared to CpG, SpA caused a modest CD19$^+$ B cell proliferation (4.14% ± 2.46% versus 19.95% ± 4.03% [Fig. 5C]). None of the other protein preparations (SpA$_{ΔX}$, SpA$_{ΔLYM}$, SpA$_{ΔLPXTG}$, rSpA, and supernatant of Δspa) induced the proliferation of purified CD19$^+$ B cells (Fig. 5C). To rule out a contribution from contaminating lipopeptides to CD19$^+$ B cell proliferation, SpA was also purified from the lgt mutant (28) (Fig. S3A). Lipoprotein diacylglycerol transferase (Lgt) catalyzes transfer of phosphatidylglycerol to the sulfhydryl moiety of a cysteine residue conserved in the signal peptides of lipoprotein precursors (29). This diacylglycerol modification modulates host immune responses (30) but is abrogated in the lgt mutant of S. aureus (28). Nonetheless, CD19$^+$ B cell expansion by SpA was independent of...
diacylglycerol modification of lipoprotein precursors catalyzed by Lgt (Fig. S3B). As observed earlier, mitogen-active SpA induced CD19+ B cells to secrete IgM but only in the presence of CD3+ T cells (Fig. 5D and E). Incubation with rSpA did not promote IgM secretion, whereas CpG-activated CD19+ B cells secreted IgM regardless of CD3+ T cells (Fig. 5D and E). SpAΔXr and SpAΔLysM also activated IgM secretion in mixed CD19+ B cell/CD3+ T cell preparations, albeit to a lesser degree than wild-type SpA without significant difference (Fig. 5D). Similar lower levels of IgM secretion were observed when CD19+ B cells/CD3+ T cells were treated with SpAΔLPXTG or with the Δspa control sample (Fig. 5D). Thus, SpA molecules are sufficient to promote proliferation of CD19+ B cells and secretion of VH3 idiotypic immunoglobulin in a CD4+ T helper cell-dependent manner. However, both the LysM domain and LPXTG sorting motif are required for optimal B cell superantigen activity.

Noncovalent peptidoglycan modification of released SpA. Cell wall hydrolases release LPXTG anchored SpA from peptidoglycan with the C-terminal structure L-Ala-D-ιGln-L-Lys(SpA-LPET-Gly5)-D-Ala-Gly4 (23). Earlier findings, and data presented here, suggest that this covalent modification is critical for the B cell superantigen activity of SpA (12). However, the LPXTG wall peptide modification does not account for the contribution of the predicted LysM domain. LysM domains have been described for proteins from bacteria, plants, and eukaryotes as independent folding units; in bacteria and plants, the LysM domain binds oligosaccharides comprised of GlcNAc and other amino sugar residues (31). To investigate the molecular basis for LysM domain-mediated B cell superantigen activity, purified SpA molecules released into the conditioned medium of cultures from S. aureus Newman and its spaΔLysM variants were cospotted with matrix and analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), focusing on signals with m/z 700 to 4,000 in the positive ion mode (Table 1). These experiments identified two main ions, m/z 1,989.24 and m/z 3,554.07, detectable in wild-type SpA. m/z 1,989.24 was interpreted as the sodium ion of (MurNAc-GlcNAc)-(AQKG5A)-(MurNAc)-(AQKG2A) (calculated m/z 1,989.90). m/z 3,554 was interpreted as (MurNAc-GlcNAc2)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A)
Several other ion signals, predicted as peptidoglycan fragments with glycan chains of 2 or 3 MurNAc-GlcNAc repeats and attached wall peptides, were also detected in wild-type SpA samples (Table 1). Importantly, none of the ion signals listed in Table 1 was observed for the SpA\textsubscript{D\textsubscript{LysM}} sample. Taken together, these experiments suggest that released SpA copurifies with noncovalently bound peptidoglycan comprised of glycan chains that are 3 to 6 amino sugar residues in length. Copurification with glycan chains was not observed for the SpA\textsubscript{D\textsubscript{LysM}} variant.

**SpA LysM binds staphylococcal peptidoglycan.** To determine whether the LysM domain of SpA alone is sufficient to bind peptidoglycan fragments, recombinant LysM (LysMSpA) and region X (XrSpA) with appended N- and C-terminal six-histidyl tags and Strep-tags were purified from the cytoplasm of \textit{E. coli} via affinity chromatography (Fig. 6A). StrepTactin-Sepharose was loaded with purified protein, washed, and used for affinity chromatography of purified staphylococcal peptidoglycan solubilized with lysostaphin, which cleaves the pentaglycine cross bridges of peptidoglycan. Columns were washed and proteins with or without bound ligand were eluted with desthiobiotin, dialyzed against water, and analyzed by MALDI-TOF MS. Compounds with \( m/z \) 2,108.75, \( m/z \) 2,335.54 and \( m/z \) 2,534.89 were identified in the eluate of the LysMSpA-peptidoglycan copurification experiment (Fig. 6B). \( m/z \) 2,108.75 has the predicted structure (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc)-(AQKA2) (calculated sodium ion \( m/z \) 2,106.95). The predicted structure of \( m/z \) 2,335.54 is (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) (calculated sodium ion \( m/z \) 2,335.04), whereas \( m/z \) 2,534.89 was designated (OAc-MurNAc-GlcNAc)-(AQKG4A2)-(OAc-MurNAc-GlcNAc)-(AQKG5A2) (calculated sodium ion \( m/z \) 2,533.38). Of note, monosaccharide (MurNAc)-

\*\*TABLE 1** MALDI-TOF analysis of released protein A from wild-type \textit{S. aureus} Newman and its variant spa\textsubscript{D\textsubscript{LysM}}

| Proposed structure\textsuperscript{a} | Predicted \( m/z \) | Observed \( m/z \) | \( \Delta \textsuperscript{c} \) | spa\textsubscript{D\textsubscript{LysM}} |
|--------------------------------------|-------------------|-------------------|----------------|------------------|
| (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc)-(AQKA2) | 1,875.85 | 1,873.64 | -2.21 | Not observed |
| (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) | 1,989.90 | 1,989.24 | -0.66 | Not observed |
| (MurNAc-GlcNAc)_2-(AQKG2A)_2 | 2,021.90 | 2,021.08 | -0.82 | Not observed |
| (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) | 2,106.95 | 2,107.05 | 0.10 | Not observed |
| (MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) | 2,192.97 | 2,191.80 | -1.17 | Not observed |
| (OAc-MurNAc-GlcNAc)_2-(AQKG2A)_2 | 2,476.36 | 2,475.29 | -1.07 | Not observed |
| (OAc-MurNAc-GlcNAc)-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) | 2,732.48 | 2,732.28 | -0.20 | Not observed |
| (MurNAc-GlcNAc)_2-(AQKG5A2)-(MurNAc-GlcNAc)-(AQKG2A2) | 2,914.09 | 2,913.98 | -0.11 | Not observed |
| (MurNAc-GlcNAc)_2-(AQKG2A2)- (MurNAc-GlcNAc)-(AQKG2A2) | 3,085.16 | 3,085.14 | -0.02 | Not observed |
| (MurNAc-GlcNAc)-(AQKG2A2)-(MurNAc-GlcNAc)-(AQKG5A2) | 3,553.34 | 3,554.07 | 1.27 | Not observed |

\textsuperscript{a}Ion signals and proposed composition of compounds from MALDI-TOF mass spectra. Ions represent sodium adducts.

\textsuperscript{b}WT, wild type.

\textsuperscript{c}\( \Delta \), the difference between predicted and observed \( m/z \).
(AQKG\textsubscript{3}A\textsubscript{2}) or disaccharide (MurNAc-GlcNAc)-(AQKG\textsubscript{3}A\textsubscript{2}) murein fragments, which are abundantly present in lysostaphin-digested peptidoglycan, were not retained during affinity chromatography on a LysM\textsubscript{SpA} column (32). As a control, peptidoglycan fragments were not retained during chromatography on an XrSpA column (Fig. 6B). Thus, the LysM domain may capture glycan chains of peptidoglycan fragments released during \textit{S. aureus} growth to implement the B cell superantigen activity of SpA together with the C-terminal peptidoglycan modification at its LPXTG motif.

**Recombinant rSpA modified with staphylococcal peptidoglycan displays mitogenic activity.** Previous studies demonstrated that a construct containing only the five IgBDs of SpA (5-IgBD) can bind \textit{V}\textsubscript{H3} idiotypic immunoglobulins and mediate cross-linking of BCRs on isolated human CD19\textsuperscript{+} B cells but cannot induce B cell expansion (12, 27, 33). When B cells were costimulated with the TLR2 agonist Pam3CSK4 or with the T cell cytokine interleukin 2 (IL-2), the 5-IgBD polypeptide promoted B cell proliferation but failed to promoted Ig production (27). rSpA used in this study was purified from the cytosol of \textit{E. coli} and is not processed by leader peptidase or sortase A (Fig. 1A). Like the 5-IgBD construct (27), rSpA cannot induce B cell expansion, immunoglobulin gene expression, or IgM secretion (Fig. 4BD). rSpA with its intact LysM and sortase cleavage site (LPET/G) is an ideal tool to evaluate the contribution of peptidoglycan ligands. Purified peptidoglycan was treated with mutanolysin, and solubilized fragments were separated by reversed-phase high-performance liquid chromatography (rpHPLC) (Fig. 7A). Fractions eluting with increasing retention times were pooled and subjected to MALDI-TOF MS (Fig. 7A and B, P1 [pool 1] and P2). Peptidoglycan fragments with \textit{m/z} 2,440.46 and 3,604.10 were identified as sodium ions of dimer (MurNAc-GlcNAc)-(AQKG\textsubscript{3}A\textsubscript{2})-(MurNAc-GlcNAc)-(AQKG\textsubscript{3}A\textsubscript{2}) (calculated \textit{m/z} 2,440.68) and trimer (MurNAc-GlcNAc\textsubscript{3}-(AQKG\textsubscript{3}A\textsubscript{2})-(MurNAc-GlcNAc)-(AQKG\textsubscript{3}A\textsubscript{2}) (calculated \textit{m/z} 3,604.97) (Fig. 7B). Of note, ion with \textit{m/z} 1,813.56 corresponded to the doubly charged version of ion with \textit{m/z} 3,604.10, and thus, only one compound from P2 could be iden-

![Graph](https://example.com/graph.png)

**FIG 7** Human B cell proliferation stimulated by rSpA bound to peptidoglycan. (A) Peptidoglycan (5 mg) prepared from \textit{S. aureus} Newman was treated with mutanolysin, and solubilized products were resolved by HPLC over a C\textsubscript{18} column by measuring absorbance at 206 nm over time. (B) Positive-ion MALDI-TOF MS identification of peptidoglycan fragments from pooled fractions shown in panel A. Pink and blue arrows depict dimer and trimer, respectively, with confirmed \textit{m/z} of 2,440.4 and 3,604.1. (C) Proliferation of negatively selected human B cells (CD19\textsuperscript{+}/CD3\textsuperscript{+}) stimulated by rSpA in PBS or rSpA preincubated with sortase A, crude peptidoglycan (PG) digest, trimer or dimer of PG as purified for panel A. Statistical analysis was performed with one-way ANOVA. ***, \( P < 0.01; ^{*}, P < 0.05.\)
tified by MALDI-TOF MS (Fig. 7B, right). rSpA was incubated with mutanolysin-treated peptidoglycan or purified dimer and trimer and sortase A. Following the removal of excess ligands and enzyme, rSpA preparations were dialyzed against buffer control and added to mixed populations of CD19⁺ B cells/CD3⁺ T cells. As expected, mock-treated rSpA resulted in negligible CD19⁺ B cell proliferation compared to that with buffer (0.80% ± 0.468% versus 0.69% ± 0.192%) (Fig. 7C). Incubation with rSpA exposed to mutanolysin-treated peptidoglycan, either the total peptidoglycan digest or pooled fractions containing peptidoglycan trimer, stimulated a small but significant proliferation of B cells (4.13% ± 0.834% and 2.39% ± 0.547%, respectively). Thus, peptidoglycan modification of rSpA results in a gain of mitogenic activity.

DISCUSSION

During cell division, S. aureus forms septal membranes and synthesizes cross wall peptidoglycan with attached SpA at midcell (34, 35). Once cross wall assembly is completed, muralytic enzymes split the peptidoglycan layer and separate two spherical cells (21, 36–39). Muralytic enzyme cleavage at cross wall peptidoglycan also provides for the surface display of cell wall-anchored SpA and for the release of peptidoglycan-linked SpA into the extracellular medium (21, 23). Early work demonstrated that S. aureus stimulates the proliferation of isolated human B cells in a SpA-dependent manner (8, 9). SpA purified from S. aureus cultures promotes B cell proliferation in mice and in human lymphocyte preparations. B cell proliferation requires direct interaction of SpA with B cell receptors as well as CD4⁺ T helper cells (10–12). Recombinant SpA (rSpA) purified from E. coli does not promote B cell proliferation, which prompted a model whereby the peptidoglycan-modified LPXTG motif must be critical for the B cell superantigen activity of SpA. In this study, we validated this model by generating an S. aureus strain producing SpA lacking its C-terminal cell wall tethering motif, SpA_DLPXTG. We completed our analysis by generating strains that produce SpA lacking the C-terminal Xr and LysM domain, SpA_DXr and SpA_DLysM, respectively. When used as a mitogen, SpA_DLysM displayed reduced activity on human B cells compared to those with SpA and SpA_DXr. We propose that this loss of activity correlates with LysM-mediated binding of peptidoglycan glycan chains. Further, some B cell superantigen activity could be restored following incubation of E. coli purified rSpA with sortase A and muralytic enzyme-treated peptidoglycan.

T cell-independent proliferation of isolated human B cells has also been reported (27). However, such B cell expansion requires large numbers of staphylococci and Toll-like receptor 2 (TLR2) activation by bacterial lipoproteins (8, 27). Such T cell-independent proliferation of human B cells may be restricted to tissues with significant pathogen burden, for example, the draining lymph nodes of deep-seated abscess lesions, where SpA cross-linking of B cell receptors may trigger staphylococcal uptake and TLR2 activation (40, 41). In contrast, localized S. aureus infection with low pathogen burden triggers systemic B cell proliferation and secretion of V₃ idiootypic immunoglobulin in humans (14). We propose that systemic modification of adaptive immune responses is associated with the release of SpA from the bacterial surface with two peptidoglycan modifications, wall peptides linked to the C-terminal LPXTG motif and glycan chains bound to the LysM domain. Released SpA molecules cross-link V₃ idiootypic BCRs to activate B cell proliferation and immunoglobulin secretion; however, this cannot be optimally accomplished without CD4⁺ T helper cells. Also required is receptor-interacting serine/threonine protein kinase 2 (RIPK2), presumably by implementing peptidoglycan signals via the nucleotide oligomerization domain 1 (NOD1) and 2 (NOD2) receptors (12, 42, 43). As NOD1 responds to muramyl peptide activation and NOD2 to wall peptides, it seems plausible that these two receptors may also be involved in perceiving peptidoglycan signals associated with SpA. Biotinylated peptidoglycan of S. aureus digested with mutanolysin has been shown to colocalize with NOD2 in primary mouse keratinocytes (44). It is unknown whether the mammalian immune system recognizes the ubiquitous polymeric glycan backbone of
peptidoglycan via NOD2 or other receptors. Similarity searches show that humans and most mammals are endowed with the evolutionarily conserved proteins LysMD1, LysMD2, LysMD3, and LysMD4 with an N-terminal lysin motif (LysM). Only LysMD3 has been studied in mice. Mouse LysMD3 is a type II integral membrane protein that colocalizes with the Golgi apparatus (45). mLysMD3-deficient mice exhibit no obvious immune response deficiencies in a number of models of infection and inflammation (45). LysM domains have been best characterized for prokaryotes, bacteriophages, and plants. LysM domains are approximately 50 amino acids long, fold with a βαβ structure, and bind to GlcNAc-containing glycans, such as chitin, chitin-like compounds, and peptidoglycan (46). In bacteriophages and bacteria, LysM domains are mostly associated with catalytic protein modules for peptidoglycan assembly and degradation (22, 46). In plants, membrane proteins with multiple LysM modules mediate immune responses to bacterial pathogens as well as symbiotic recognition of bacteria (47, 48).

Future studies need to determine how B cells perceive the peptidoglycan signals of SpA, for example, following endocytosis of BCRs with cross-linked SpA, and to define the specific contributions of T cells to B cell proliferation and immunoglobulin secretion. In summary, this work provides insight into the mechanisms whereby localized infection with S. aureus and release of peptidoglycan-modified SpA trigger systemic B cell responses that divert adaptive immune responses to prevent the establishment of protective immunity.

MATERIALS AND METHODS

Bacterial strains and growth. S. aureus strains Newman and RN4220 and variants were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 37°C. Escherichia coli strains DH5α and BL21(DE3) were grown in Luria-Bertani broth (LB) or agar at 37°C. Ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) were used for the selection of pET-15b and pKOR1 derivatives in E. coli and S. aureus, respectively (49). Erythromycin (10 μg/ml) was used to select for ermB-marked mutations in S. aureus (50). Additional cloning procedures for the generation of spa strain variants are provided in the supplemental material, along with methods to examine the production and localization of SpA in the envelope of bacterial cells (Text S1; Table S1).

Animal experiments. Animal experiments were conducted in accordance with institutional and federal guidelines following experimental protocol review and approval and supervision by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago. Overnight cultures of bacteria were diluted 1:100 into fresh TSB and grown at 37°C with rotation to an A600 of 0.4. Staphylococci were sedimented by centrifugation at 13,000 × g for 5 min, washed, and suspended in PBS to an A600 of 0.4. Staphylococcal challenge doses (0.1-ml suspensions of bacteria in PBS) were quantified by spreading of sample aliquots on TSA plates, incubation, and enumeration of CFU. Cohorts (n = 10) of 6-week-old BALB/c mice were anesthetized via intraperitoneal injection of anesthetic (64.2 mg/ml of ketamine and 4.9 mg/ml of xylazine per kg of body weight). Anesthetized mice were infected by injection of 0.1 ml of 1 × 108 CFU S. aureus into the periorbital venous sinus of the right eye. Mice were anesthetized and bled with heparin-coated capillary tubes from periorbital venous sinuses on days 5, 12, and 19 or by terminal cardiac puncture (day 26). Coagulated blood specimens were centrifuged at 10,000 × g; serum supernatant was retrieved for measurements of V3 Xr,3 clonal IgG and IgM contents using ELISA (see Text S1 for details on ELISA). To ensure reproducibility, mouse experiments were repeated.

Preparation of test articles used in B cell proliferation assays. Details for B cell isolation and proliferation assays are provided in the supplemental material (Text S1). To examine the effect of killed bacteria on B cell proliferation, overnight cultures of S. aureus were diluted 1:100 into fresh TSB and grown at 37°C to an A600 of 0.4. For tet-spa and tet-spa::ermB strains, 80 ng/ml and 400 ng/ml of anhydrotetracycline were added to cultures, respectively. Staphylococci were centrifuged, washed, and normalized to a load of 4 × 106 CFU/ml. Bacteria were killed by addition of 0.5% formaldehyde and incubation at room temperature for 3 h, followed by heat treatment at 80°C for 3 min (9). Killed staphylococci were centrifuged for 5 min at 13,000 × g, the supernatant was discarded, and bacteria were suspended in the same volume of PBS. To examine the effect of purified SpA on B cell proliferation, first the sbi::erm allele was crossed in each background, yielding S. aureus sbi::ermB (isogenic wild type lacking Sbi), spa::ermB, spa::ermB, spa::ermB, spa::ermB, spa::ermB, spa::ermB, spa::ermB, and spa::ermB variants, which were grown overnight in TSB and diluted 1:100 into 8 liters of fresh TSB. Cultures were grown with rotation at 37°C for 3 h and centrifuged at 8,000 × g for 10 min. Culture supernatants were filtered through 0.22-μm membranes (Millipore) and subjected to affinity chromatography over 2 ml of IgG Sepharose 6 Fast Flow (GE Healthcare). Bound proteins were eluted with 0.1 M glycine-HCl (pH 3.0), followed by immediate pH neutralization with 1 M Tris-HCl (pH 8.0). Eluates were concentrated in a vacuum centrifuge concentrator, dialyzed against PBS, and used at a concentration of 80 nM in B cell proliferation assays. Protein concentrations were determined with the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).
Purification of peptidoglycan and recombinant proteins and binding experiments. Peptidoglycan was prepared as described previously (51), suspended in water, normalized to an A_260 of 10, and stored at –20°C. Recombinant proteins were produced using E. coli BL21(DE3) from crude lysate generated as described previously (26, 51, 52). Additional details are provided in the supplemental material (Text S1).

Statistical analysis. Statistical analysis was performed with GraphPad Prism (GraphPad Software Inc., La Jolla, CA; version 7.04). Statistically significant differences were calculated by using statistical methods as indicated. P values of ≤0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

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