Children with Invasive *Staphylococcus aureus* Disease Exhibit a Potently Neutralizing Antibody Response to the Cytotoxin LukAB

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Despite the importance of *Staphylococcus aureus* as a common invasive bacterial pathogen, the humoral response to infection remains inadequately defined, particularly in children. The purpose of this study was to assess the humoral response to extracellular staphylococcal virulence factors, including the bicomponent leukotoxins, which are critical for the cytotoxicity of *S. aureus* toward human neutrophils. Children with culture-proven *S. aureus* infection were prospectively enrolled and stratified by disease type. Fifty-three children were enrolled in the study, of which 90% had invasive disease. Serum samples were obtained during the acute (within 48 h) and convalescent (4 to 6 weeks postinfection) phases, at which point both IgG titers against *S. aureus* exotoxins were determined, and the functionality of the generated antibodies was evaluated. Molecular characterization of clinical isolates was also performed. We observed a marked rise in antibody titer from acute-phase to convalescent-phase sera for LukAB, the most recently described bicomponent leukotoxin. LukAB production by the isolates was strongly correlated with cytotoxicity in vitro, and sera containing anti-LukAB antibodies potently neutralized cytotoxicity. Antibodies to *S. aureus* antigens were detectable in healthy pediatric controls but at much lower titers than in sera from infected subjects. The discovery of a high-titer, neutralizing antibody response to LukAB during invasive infections suggests that this toxin is produced in vivo and that it elicits a functional humoral response.

**MATERIALS AND METHODS**

**Patient enrollment.** This was a prospective cohort study of children (between 6 months and 18 years of age) admitted to the Monroe Carell Jr. Children’s Hospital at Vanderbilt with culture-confirmed *S. aureus* infection identified within the first 5 days of hospitalization. Potential study subjects were identified through daily contact with the Pediatric Infectious Diseases and Hospital Medicine inpatient services from October 2010 to June 2012. Informed consent was obtained, and children were screened for the following exclusion criteria: polymicrobial infection, primary or secondary immune compromise (including long-term oral or parenteral corticosteroids), history of (or current) malignancy, receipt of intravenous immunoglobulin (IVIG) or blood products in the past 12 months, and known history of invasive staphylococcal disease (Fig. 1). Serum samples were obtained immediately upon enrollment in the study (acute-phase sera) and 4 to 6 weeks following enrollment (convalescent-phase sera). Sera were obtained by centrifugation of unheparinized whole blood samples, and sera were stored at −20°C until processing. When available, clinical isolates were also obtained for molecular characteriza-
tion. The study was approved by the Vanderbilt University Medical Center (VUMC) Institutional Review Board.

Healthy control (HC) subjects were recruited from two sources: from an ongoing clinical trial at VUMC in which blood was obtained from healthy siblings of diabetic children for genetic analysis and from same-day surgery at the Monroe Carell Jr. Children’s Hospital at Vanderbilt from subjects undergoing outpatient surgical procedures for noninfectious diagnoses. Healthy controls were selected if they met the following inclusion criteria: age between 6 months and 18 years, no known history of S. aureus disease of any type, no known primary or secondary immune compromise (including long-term oral or parenteral corticosteroids), no history of (or current) malignancy, and no receipt of IVIG or blood products in the past 12 months. Serum samples were obtained from healthy control subjects at the time of enrollment.

Serum antibody measurement by enzyme-linked immunosorbent assay (ELISA). The serum antibody response was measured against 11 specific S. aureus exotoxins: α-hemolysin (Hla), V8 protease, and each subunit of the bicomponent leukotoxins LukAB (LukA and LukB), PVL (LukS-PV and LukF-PV), LukED (LukE and LukD), and the gamma-hemolysins (HlgA, HlgB, and HlgC). Recombinant Hla and V8 protease were purchased in purified form and reconstituted per manufacturer specifications (Sigma). All other toxins were expressed and purified as described previously (8, 14, 15).

Optimal exotoxin concentration for ELISA was determined by crisscross dilution, and indirect ELISA for each antigen was performed as previously described (16, 17). Briefly, purified antigens were diluted in phosphate-buffered saline (PBS) to a concentration of 0.5 μg/ml and bound to 96-well ELISA plates overnight. Wells were then aspirated and blocked at room temperature (RT) for 1 h with 5% nonfat dried milk in Tris-buffered saline. Serial 2-fold dilutions of sera were added to the plate and incubated for 2 h at RT. After plates were washed, horseradish peroxidase (HRP)-conjugated murine monoclonal antibodies against human total IgG diluted 1:1,000 were added, and plates were incubated at RT for 2 h. Next, substrate solution (3,3′,5,5′-tetramethylbenzidine) was added, and plates were incubated at RT. The reaction was stopped at 30 min with 2 M sulfuric acid, and plates were read spectrophotometrically at 450 nm. ELISA titers were measured as the highest serum dilution at which the absorbance value was at least three times the background absorbance of uncoated control wells. Serum depleted of IgG was used as a negative control, and rabbit IgG (with goat-derived HRP-conjugated anti-rabbit IgG) was used to control for nonspecific binding. Samples were run in duplicate, independently on separate days, and a third run was performed if the first two titer values differed by one dilution. Antibody titer results are presented as geometric mean titers (GMT) for a given population unless otherwise specified.

Molecular characterization. Initial determination of methicillin resistance in S. aureus strains was made by the VUMC clinical laboratory. For methicillin-resistant S. aureus (MRSA) isolates, confirmation of methicillin resistance was performed by plating samples onto paired manitol salt agar plates, with and without 4 μg/ml of oxacillin (Hardy Diagnostics, Santa Maria, CA). After incubation at 37°C for 48 h and at room temperature for 18 h, plates were inspected for yellow colonies indicative of manitol fermentation, a characteristic of S. aureus. After subculturing onto tryptic soy agar with 5% sheep blood (Hardy Diagnostics), rapid latex agglutination testing for clumping factor and protein A was performed on all isolates (Staphaurex Plus; Remel). Following phenotypic confirmation of S. aureus, crude genomic DNA was prepared by incubating the isolates with lysostaphin for 1 h at 37°C and heating the samples at 95°C for 15 min. This template DNA was used to detect the presence of the mecA gene, using previously described oligonucleotide primer sequences (18).

Genomic DNA was purified (Wizard SV purification system; Promega, Madison, WI) and used as the template for PCR detection of genes encoding pvl (19), the accessory gene regulator (agr) locus type (20), and lukAB (12). Assignment of staphylococcal cassette chromosome mec (SCCmec) type by ccr and mec complex typing was performed as previously described (21). Repetitive-element, sequence-based PCR (DiversiLab System; bioMérieux) was used to determine strain type and genetic relatedness between strains (22).

Toxin neutralization assays. Human promyelocytic HL-60 cells were cultured in RPMI 1640 (Cellgro), 10% heat-inactivated fetal bovine serum (FBS), and 100 μg/ml penicillin-100 μg/ml streptomycin (Pen/Strep; Cellgro) and allowed to differentiate to neutrophil-like cells for 3 days per standard techniques (23). S. aureus strain Newman was cultured from a single colony in 3 ml of RPMI and Casamino Acids (RPMI-CAS) and grown at 37°C with shaking at 180 rpm. After 24 h, the strain was subcul-
tured 1:100 in 5 ml of RPMI-CAS in a 15-ml tube and cultured for 5 h at 37°C with shaking at 180 rpm. This growth condition is known to stimulate LukAB production (8, 12). Culture filtrates were obtained by centrifugation at 4,000 rpm at 4°C for 15 min with filter sterilization using 0.2-μm-pore-size syringe filters. Supernatants were tested for baseline toxicity against PMN-HL60s. Dose response of supernatant toxicity was assessed by adding supernatant to the cell culture starting at 20% culture supernatant and diluting 2-fold 8 times in 20 μl RPMI-CAS, with a goal 50% lethal dose (LD₅₀) of ~2.5%.

To measure the neutralization capacity of patient sera, 10 μl of each serum sample was added in triplicate to 96-well plates. Toxin supernatant diluted 1:1 with RPMI-CAS was added, 10 μl/well, and wells were mixed by pipetting and incubated for 30 min at RT. Wells containing only RPMI-CAS medium served as an unintoxicated control, while culture supernatant alone, sans patient sera, served as a positive toxicity control. PMN-HL60 cells were added to the plate at 80 μl/well (1 × 10⁶ cells/well) and mixed with the serum/supernatant mixture. This mixture was incubated at 37°C and 5% CO₂ for 1 h, and CellTiter (Promega) was added to 10 μl/well to measure cell viability/metabolism. After 2 h of incubation with CellTiter at 37°C and 5% CO₂, plates were read spectrophotometrically at 490-nm absorbance to measure cell survival. Neutralization titers of patient sera were determined by incubating the above-described mixture with serial dilutions of sera beginning with a 1:100 dilution, and the titer was considered neutralizing if ≥70% of the cells were alive at that dilution.

Isolation of primary human PMNs. Blood samples were obtained from anonymous, consenting, healthy adult donors asuffy coats from the New York Blood Center. This study was reviewed and approved by the New York University Langone Medical Center Institutional Review Board.

PMNs were isolated from peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque PLUS gradient. The pellets were subsequently washed with PBS, and PMNs were separated from erythrocytes with 3% dextran (dextran 500; Pharmacosmos) in 0.9% sodium chloride. Remaining erythrocytes were lysed with ACK lysis buffer (Gibco). PMN purity was at 90 to 95% as determined by flow cytometry.

Cytotoxicity of isolates from children with invasive S. aureus infection. Culture filtrates from S. aureus strains responsible for children’s invasive infection were generated as described above. Freshly purified PMNs were plated in 96-well black bottom tissue culture-treated plates (Corning) at 1 × 10⁵ cells/well in a final volume of 100 μl of phenol red-free RPMI (Gibco) supplemented with 10% heat-inactivated FBS. Cells were intoxicated with 10% and 5% (vol/vol) culture filtrates for 1 h at 37°C and 5% CO₂. Controls for 100% viability were composed of PMNs with S. aureus growth medium (RPMI-CAS), while controls for 100% PMN lysis included the addition of Triton X-100 (0.1%) in RPMI-CAS.

To evaluate PMN viability following intoxication, 50 μl of supernatant was removed and added to wells containing 50 μl of LDH reagent (Cytotox-ONE homogeneous membrane integrity assay, Promega) and incubated for an additional 10 min at RT. Fluorescence was measured using a PerkinElmer Envision 2103 multilabel reader (excitation, 555 nm; emission, 590 nm), and data were normalized to 100% PMN lysis.

Ex vivo infections with S. aureus associated with pediatric infections. S. aureus-mediated killing of PMNs by extracellular bacteria was determined as previously described (7, 8). Briefly, clinical S. aureus strains were normalized to an optical density at 600 nm (OD₆₀₀) of 1.0, which represents approximately 1.0 × 10⁹ CFU/ml, using a spectrophotometer (Genesys 20; Thermo Scientific) following subculture. PMNs were plated at 1 × 10⁵ cells/well and were infected with a multiplicity of infection (MOI) of 10 (10 S. aureus bacteria per PMN) for 1 h at 37°C and 5% CO₂. To evaluate the integrity of host cell plasma membranes following intoxication, we employed SYTOX green (Invitrogen) as previously described (12). Each well was mixed with 100 μl of PBS and SYTOX green (0.1 μM) and incubated at room temperature in the dark for 10 min.

Neutralization of USA300-mediated killing of primary PMNs. USA300 community-associated MRSA (CA-MRSA) strain LAC was normalized to an OD₆₀₀ of 1.0 following subculture, as described above. PMNs were plated at 1 × 10⁵ cells/well and were infected with an MOI of 10 in the presence of different dilutions of sera collected from acute-phase, convalescent-phase, or healthy control children for 1 h at 37°C and 5% CO₂. Membrane disruption was evaluated using SYTOX green as described above.

Immunoblot detection of toxins produced by S. aureus associated with pediatric infections. Culture filtrates from S. aureus-RPMI-CAS-grown bacteria, the same used in the intoxication assay, were precipitated with 10% trichloroacetic acid (TCA) (vol/vol). The precipitated proteins were washed once with 100% ethanol, air dried, resuspended with 30 μl of SDS-Laemmli buffer, and boiled at 95°C for 10 min. Precipitated exoproteins were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antibodies against LukA (12) and alpha-toxin (Sigma), which were detected with Alexa Fluor-680-conjugated anti-rabbit secondary antibody (Invitrogen). Membranes were scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

Statistical analysis. To satisfy normality assumptions, ELISA titers were log₁₀-transformed for statistical analysis, and antibody titers against each specific antigen were reported as the geometric mean titer. Geometric mean titers were compared using an independent t test to make comparisons between populations (e.g., MRSA versus methicillin-susceptible S. aureus [MSSA], acute-phase invasive disease versus healthy controls), and a paired t test was used to make comparisons between acute-phase and convalescent-phase values. These analyses were restricted to subjects with invasive disease, due to the small number of subjects in the cohort with skin or soft tissue infections (SSTI; n = 5) and the inability to draw statistically significant conclusions from this segment of the cohort. Differences were estimated via ratios of geometric means with corresponding 95% confidence intervals and P values. Correlations between toxin production and cytotoxicity were assessed by calculating a Spearman’s correlation coefficient. All data analyses were performed using an R value of 2.15.

RESULTS

Characteristics of the patient population. During the study period, 102 children were identified with culture-proven S. aureus disease within the first 5 days of their hospitalization. Of these, 48 were excluded from this study based on the exclusion criteria (Fig. 1). Of the remaining 54, one patient’s legal guardian declined consent, and the remaining 53 children were enrolled in the study. The mean age of children enrolled in the study was 6.7 years (standard deviation [SD], 4.8 years), and 60% were male.

Twenty-six patients were being treated for osteomyelitis or septic arthritis, 19 for bacteremia/sepsisemia, 4 for pneumonia, and 5 with skin or soft tissue infection. Children with osteomyelitis, septic arthritis, pneumonia, and bacteremia/sepsisemia were stratified as “invasive disease,” and those with skin or soft tissue infections were classified as “noninvasive disease.” The mean age of healthy control subjects was 7.6 years (SD, 4.7 years), and 52% were male.

Molecular characteristics of S. aureus isolates. Nearly half of the S. aureus isolates (48%) were methicillin resistant. The majority of clinical isolates (53%) were classified as the USA300 pulse type. The second-most-common pulse type was USA500 (23%), followed by USA100 (16%). The majority of clinical isolates (87%) possessed agr type I, and the remainder carried agr type II. All of the isolates were positive for lukAB by PCR, and 59% of the isolates were pvI positive. All of the MRSA isolates possessed SCCmec type IV.
Acute- and convalescent-phase antibody response to S. aureus disease. For the majority of the extracellular proteins tested, there was a statistically significant rise in titer from acute- to convalescent-phase disease, including for LukA, LukB, alpha toxin (Hla), V8 protease (V8P), the two subunits of PVL (LukF-PV and LukS-PV), and the two subunits of LukED (LukE and LukD) (Fig. 2). The bicomponent subunits LukF-PV, LukE, HlgB, and LukPV were significantly higher than the titer in the acute phase for LukA, LukB, Hla, LukF-PV, LukD, and HlgA. The paired t-test was used to determine statistical significance of the rise in acute-phase to convalescent-phase titers for each antigen. Subjects for which a paired sample was not obtained (e.g., no convalescent-phase sample was available) are included in the overall GMT displayed in the figure but were censored from the acute:convalescent statistical analysis shown in Table 1.

Comparison with healthy controls. To contrast the antibody response generated by disease to baseline levels in the healthy population, acute-phase and convalescent-phase sera from children with invasive S. aureus disease were compared to those from healthy pediatric controls. A low-level antibody response was detected against all antigens tested in sera from healthy controls (Fig. 2).

Titers from acute-phase sera following invasive disease were significantly higher for all antigens measured than those from healthy controls, with the exception of LukD (2.6-fold difference in acute:control GMT, 95% confidence interval [CI] of the ratio of 0.64 to 10.9, P = 0.18). Geometric mean titers from convalescent-phase sera were also significantly higher for all antigens measured than those from HC, again with the exception of LukD (3.4-fold difference in convalescent:control GMT, 95% CI of the ratio of 0.8 to 14.0, P = 0.09).

Titers from sera following noninvasive disease (skin and soft tissue infection) did not differ from healthy control sera for any antigen tested (data not shown).

LukAB versus PVL. As 41% of the clinical isolates in this study were PVL negative, we repeated the analyses after excluding PVL-negative isolates from the data set (Fig. 3). Here, geometric mean

![FIG 2 Geometric mean IgG titers in serum from healthy pediatric controls and acute- and convalescent-phase sera from children with invasive S. aureus disease. These target the two subunits of LukAB (LukA and LukB), alpha hemolysin (Hla), V8 protease (V8P), the two subunits of PVL (LukF-PV and LukS-PV), and the two subunits of LukED (LukE and LukD). Samples were run in duplicate, independently on separate days, and a third run was performed if the first two titer values differed by one dilution. *, the convalescent IgG response was significantly higher than the titer in the acute phase for LukA, LukB, Hla, LukF-PV, LukD, and HlgA. The paired t-test was used to determine statistical significance of the rise from acute-phase to convalescent-phase titers for each antigen. Subjects for which a paired sample was not obtained (e.g., no convalescent-phase sample was available) are included in the overall GMTs displayed in the figure but were censored from the acute:convalescent statistical analysis shown in Table 1.

![FIG 3 Anti-LukAB and anti-PVL responses when restricting to PVL-positive isolates, by geometric mean titer. *, the convalescent-phase IgG response against each subunit of PVL (LukF-PV and LukS-PV) was significantly higher than the acute titer, when restricting the analysis to PVL-positive isolates (paired t test, P < 0.05). Further, PVL-F and PVL-S antibody responses were significantly higher when restricting the analysis to PVL-positive isolates alone (P < 0.01 for the difference in GMT, Wilcoxon signed-rank test), a difference that was not present for anti-LukA and LukB responses. Samples were run in duplicate, independently on separate days, and a third run was performed if the first two titer values differed by one dilution.](http://iai.asm.org/ on October 2, 2017 by guest)

TABLE 1 Humoral response to S. aureus exoproteins in paired acute-phase and convalescent-phase sera following invasive disease

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GMT, acute-phase serum</th>
<th>GMT, convalescent-phase serum</th>
<th>Fold increase, convalescent:acute</th>
<th>95% CI of the ratio</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LukA</td>
<td>314.2</td>
<td>735.1</td>
<td>2.34</td>
<td>1.65–3.33</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>LukB</td>
<td>108.8</td>
<td>350.7</td>
<td>3.22</td>
<td>1.81–5.72</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Hla</td>
<td>165.7</td>
<td>327.0</td>
<td>1.97</td>
<td>1.29–3.01</td>
<td>0.002</td>
</tr>
<tr>
<td>V8 protease</td>
<td>39.3</td>
<td>68.7</td>
<td>1.75</td>
<td>1.33–2.3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>LukF-PV-S</td>
<td>39.3</td>
<td>58.6</td>
<td>1.49</td>
<td>0.95–3.33</td>
<td>0.07</td>
</tr>
<tr>
<td>LukPV-F</td>
<td>129.0</td>
<td>228.3</td>
<td>1.77</td>
<td>1.12–2.81</td>
<td>0.01</td>
</tr>
<tr>
<td>LukD</td>
<td>25.0</td>
<td>67.4</td>
<td>2.69</td>
<td>1.26–5.62</td>
<td>0.01</td>
</tr>
<tr>
<td>LukE</td>
<td>19.9</td>
<td>30.0</td>
<td>1.51</td>
<td>0.89–2.52</td>
<td>0.126</td>
</tr>
<tr>
<td>HlgA</td>
<td>151.4</td>
<td>324.0</td>
<td>2.14</td>
<td>1.53–4.17</td>
<td>0.0001</td>
</tr>
<tr>
<td>HlgB</td>
<td>32.8</td>
<td>41.6</td>
<td>1.27</td>
<td>0.80–2.11</td>
<td>0.17</td>
</tr>
<tr>
<td>HlgC</td>
<td>25.0</td>
<td>30.8</td>
<td>1.23</td>
<td>0.73–1.98</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Acute-phase sera for which a convalescent pair was not obtained were excluded from this portion of the analysis.

a Paired t-test used to determine statistical significance of the rise in acute-phase to convalescent-phase titer for each antigen.
titers for each subunit of PVL were significantly higher than in the overall cohort (348.9 for LukS-PV and 92.4 for LukF-PV in the restricted analysis compared to 228.4 and 59.1, respectively, in the overall cohort) but not for the subunits of LukAB. Even when restricting to PVL-positive isolates, the anti-LukA and anti-LukB antibody responses exhibited a 3.5-fold increase in titer from acute-phase to convalescent-phase sera, compared with a 2-fold increase in titer against each subunit of PVL.

**MSSA compared to MRSA.** Sera from patients with MSSA disease were directly compared to sera from those infected with MRSA. Following invasive disease, the GMT for every antigen tested was lower for those with MSSA disease than for those with MRSA. Following invasive disease, the GMT for every antigen tested was lower for those with MSSA disease than for those with MRSA. The GMT for every antigen tested was lower for those with MSSA disease than for those with MRSA.

**Correlation of in vitro cytotoxicity with toxin production.** In order to assess the correlation between secretion of extracellular toxins and cytotoxicity *in vitro*, primary human polymorphonuclear cells (PMNs; or neutrophils) were incubated with culture filtrates from the clinical isolates obtained from children with invasive staphylococcal disease [Fig. 4](#fig4). Exoproteins from the large majority of the clinical isolates were cytotoxic toward PMNs, although 5 strains showed impaired killing of PMNs [Fig. 4A](#fig4A). Consistent with our previous observation that LukAB is the main cytotoxin present in culture filtrates of *S. aureus* [12](#12), the majority of strains produced LukAB in *vitro* [Fig. 4B](#fig4B). The secretion of LukAB was strongly correlated with cytotoxicity *in vitro* (P < 0.0001, Spearman correlation coefficient = 0.76).

**Toxin neutralization.** We performed a functional assessment of the anti-LukAB antibody response by measuring the cytoprotective effect of patient sera when incubated with supernatant from *S. aureus* strain Newman. We have previously demonstrated that strain Newman secretes high levels of LukAB and comparably low levels of other leukotoxins, and deletion of lukAB in strain Newman completely eliminates the cytotoxic activity [12](#12). In this cohort, acute-phase sera neutralized the cytotoxicity of *S. aureus* supernatant [Fig. 5](#fig5), with a geometric mean neutralization titer (GMNT) of 578, a 3-fold increase in the neutralization capacity compared to that of healthy control serum (GMNT of healthy control serum = 194, 95% CI of the ratio of 1.2 to 7.5, P < 0.05). Convalescent-phase sera neutralized LukAB-mediated toxicity more potently than either acute-phase sera or healthy control sera (Fig. 5; see also Fig. S1 in the supplemental material). The GMNT for convalescent-phase sera was 1,670, an 8.6-fold increase compared to that for healthy controls (95% CI of the ratio of 3.3 to 22.1, P < 0.001) and a 3.4-fold increase in GMNT compared to acute-phase sera (95% CI of the ratio of 2.3 to 5.0, P < 0.001). For both acute-phase and convalescent-phase sera, neutralization titers correlated with serum IgG titers by ELISA (P < 0.01 for both groups).

**Neutralization of USA300 cytotoxicity.** To further evaluate the breadth of neutralizing activity for the sera from the pediatric cohort, we tested the sera in an *ex vivo* infection assay where primary PMNs where challenged with a USA300 strain in the presence of different concentrations of acute-phase, convalescent-phase, and healthy control sera. Similar to the experiment using Newman culture filtrates, the acute-phase and convalescent-phase sera were more potent at neutralizing USA300-mediated PMN membrane damage than healthy control sera [Fig. 6](#fig6). In addition, we observed a modest but significant increase in protection by the convalescent-phase sera.

**DISCUSSION**

The major findings of this study are that children with invasive *S. aureus* disease exhibit a high-titer, neutralizing antibody response to LukAB, and the rise in titer from acute disease to convalescence provides strong evidence of LukAB expression and production during invasive human disease. To our knowledge, this is the first description of the humoral immune response to LukAB, a recently described leukotoxin that is both abundantly produced by *S. aureus* and critical to *S. aureus* ability to subvert human PMNs [7, 8, 12, 13, 24, 25]. LukAB was ubiquitously present in clinical isolates from this cohort of children with invasive *S. aureus* disease, and the results of this study support the prominence of this toxin in the *S. aureus* proteome during human disease.

LukAB is increasingly recognized as a critical component of the *S. aureus* virulence repertoire devoted to evasion of human PMNs, although its exact role *in vivo* remains to be fully elucidated. Previous studies have shown that *S. aureus*, both MSSA and MRSA, kills human phagocytes in an LukAB-dependent manner [12, 13] and that disruption of lukAB markedly impairs the ability of *S. aureus* to avoid whole blood and PMN-mediated killing [7, 8, 12]. Notably, deletion of lukAB in different *S. aureus* strains has a more prominent effect on PMN survival than the deletion of any other bicomponent leukotoxin [7, 8, 12]. The prominent role of LukAB in the ability of *S. aureus* to target and kill PMNs, the first and often most critical line of defense to infection [26], is likely due to the increased expression of this toxin upon PMN encounter [8] and the fact that this toxin exploits CD11b as its receptor [7], a highly abundant protein on the surface of neutrophils.

This is one of the largest known studies involving paired (acute and convalescent) sera of children with invasive staphylococcal disease. The pediatric population confers several advantages in a study of the humoral response to *S. aureus* infection. First, extended carriage of *S. aureus* exerts an unknown effect on the serologic profile of specific antistaphylococcal antibodies. The pediatric serologic profile, lacking a lifetime of intermittent or persistent *S. aureus* nasal colonization, may more directly reflect the response to acute infection. Second, children with staphylococcal disease have uniquely defined phenotypes of invasive disease (e.g., acute hematogenous osteomyelitis and septic arthritis), allowing for more straightforward stratification of disease types. Third, the

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**TABLE 2 Comparison of sera following acute invasive disease, MSSA versus MRSA**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GMT, MSSA</th>
<th>GMT, MRSA</th>
<th>Fold difference</th>
<th>95% CI of the ratio</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LukA</td>
<td>252.1</td>
<td>572.5</td>
<td>0.44</td>
<td>0.12–1.35</td>
<td>0.19</td>
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<tr>
<td>LukB</td>
<td>51.4</td>
<td>295.9</td>
<td>0.17</td>
<td>0.04–0.79</td>
<td>0.025</td>
</tr>
<tr>
<td>Hla</td>
<td>164.9</td>
<td>188.7</td>
<td>0.78</td>
<td>0.19–3.19</td>
<td>0.72</td>
</tr>
<tr>
<td>V8 protease</td>
<td>23.8</td>
<td>106.7</td>
<td>0.22</td>
<td>0.06–0.87</td>
<td>0.032</td>
</tr>
<tr>
<td>LukPV-F</td>
<td>35.2</td>
<td>50.3</td>
<td>0.87</td>
<td>1.36–3.8</td>
<td>0.092</td>
</tr>
<tr>
<td>LukPV-S</td>
<td>59.0</td>
<td>184.3</td>
<td>0.32</td>
<td>0.04–1.3</td>
<td>0.092</td>
</tr>
<tr>
<td>LukD</td>
<td>13.9</td>
<td>68.0</td>
<td>0.2</td>
<td>0.03–1.21</td>
<td>0.08</td>
</tr>
<tr>
<td>LukE</td>
<td>12.7</td>
<td>95.6</td>
<td>0.13</td>
<td>0.03–0.69</td>
<td>0.018</td>
</tr>
<tr>
<td>HlgA</td>
<td>120.5</td>
<td>167.4</td>
<td>0.72</td>
<td>0.31–2.66</td>
<td>0.66</td>
</tr>
<tr>
<td>HlgB</td>
<td>30.4</td>
<td>38.0</td>
<td>0.80</td>
<td>1.6–3.23</td>
<td>0.79</td>
</tr>
<tr>
<td>HlgC</td>
<td>19.2</td>
<td>29.6</td>
<td>0.65</td>
<td>0.09–1.67</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*P value determined using independent t test.

Samples were run in duplicate, independently on separate days, and a third run was performed if the first two titer values differed by one dilution.

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relative lack of medical comorbidities and the known age-related dysregulation of humoral immune responses and reduced lymphocytes in the elderly (27, 28), potentially altering the immune response to *S. aureus*, are advantages to the study of the pediatric population.

Pediatric serologic studies to date have focused primarily on the response to PVL (29, 30), a bicomponent pore-forming toxin that targets neutrophils and is epidemiologically linked to epidemic strains of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) (19, 31). Hermos et al. measured antibody levels in 43 children with SSTI and 23 children with invasive disease (32). As in our study, they found that antibodies to PVL were detectable at low levels in healthy controls but were higher following disease. Neutralizing antibody to PVL did not appear to protect against SSTI, and the protective effects of antibodies against LukAB or other members of the bicomponent leukotoxin family have not been evaluated. A recent study by Fritz et al. measured the humoral response to alpha toxin and PVL in 56

**FIG 4** Expression of LukA in clinical isolates from children with invasive *S. aureus* disease is correlated with cytotoxicity toward human neutrophils. (A) The majority of isolates showed cytotoxicity at 10% and 5% culture filtrate (vol/vol) after a 1-h incubation, as measured by LDH release from primary human neutrophils. Independent colonies from each isolate were used to start broth cultures, where culture filtrates were individually tested in duplicate. Data represent the means ± standard errors of the means (SEM) of LDH release of neutrophils from 6 human donors. (B) The majority of clinical isolates produced LukA and alpha toxin *in vitro*, as evaluated by SDS-PAGE separation and immunoblotting. Immunoblots were repeated with independent culture filtrates that showed the same results. Expression of LukA *in vitro* was significantly correlated with cytotoxicity, *P* < 0.001. Spearman correlation coefficient = 0.76.
children with invasive \textit{S. aureus} disease, as well as 167 with non-invasive disease. The authors concluded that antibodies against alpha toxin were protective against future infection (24); however, antibody titers were not presented. In addition, the functional capacity of the antibodies to neutralize alpha toxin was not evaluated. Further, LukAB was not examined in this study, and future work in this area should focus on whether the generation of high-titer, neutralizing antibodies to LukAB alters subsequent disease risk.

Antibodies to \textit{S. aureus} antigens were detectable in healthy pediatric controls in this study, but at much lower titers than infected subjects. This is consistent with previous data suggesting that colonization may result in a low-level humoral response to staphylococcal antigens in children and adults (33–35), although the bicomponent leukotoxins were not reported in these previous studies. The role of colonization as an immunizing event remains unclear, as colonization appears to confer an increased risk of staphylococcal disease but may reduce mortality (36, 37). Antibody titers following noninvasive disease were not distinguishable from levels found in healthy controls. Since the majority of subjects in this study of hospitalized children had invasive disease, the study was not powered to detect statistically significant differences when comparing those with noninvasive disease to other groups.

Given reported differences in the severity of MRSA disease compared with MSSA disease (38), we compared the humoral response to infection stratified by methicillin resistance. Geometric mean titers against several exotoxins were significantly higher following MRSA disease than with MSSA disease. As the isolates were from a variety of genetic lineages, this difference is unlikely to be related to clonal relatedness of strains. This finding may be partially explained by recent data suggesting that the Agr regulatory system in epidemic CA-MRSA strains led to hyperexpression of extracellular toxins and exoenzymes (39, 40). In children, invasive MRSA infection is associated with higher C-reactive protein levels and prolonged erythrocyte sedimentation rate, markers of a heightened inflammatory state (38, 41). The exuberant immune response stimulated by MRSA isolates compared to that by MSSA may have contributed to these serologic differences.

One caveat to our study is that culture-proven \textit{S. aureus} infection, which occurs at a time point that is not reached until several days into the disease process, was required for inclusion in the cohort. Thus, the acute-phase sera collected do not represent true baseline levels of antistaphylococcal antibodies. Analysis of the healthy control population, however, provides for comparison to the presumed baseline state. Additionally, convalescent-phase sera were collected 4 to 6 weeks after enrollment. Longitudinal data documenting persistence of this IgG response are lacking, and we plan to reassess the serologic profiles in these subjects at 1 year postinfection or longer. It should also be noted that the healthy control cohort was comprised of nonhospitalized subjects, a potential confounding factor, as the infected cases are all hospitalized patients with acute illness. It would not be expected, however, that hospitalization alone would alter the antistaphylococcal serologic profile, and thus the differences observed are likely to be a result of acute disease. Further, our sample size was not large enough to draw statistically significant conclusions regarding correlations between clinical disease severity and specific antibody titers, and this will be a focus of future work. Finally, \textit{in vitro} neutralization data are not meant to serve as a surrogate marker of protection \textit{in vivo} but rather as a measure of the functional activity of anti-LukAB antibodies. Work is ongoing to assess the \textit{in vivo} protective capacity of this antibody response.

The LukAB locus was present in all clinical isolates in this study of children with invasive \textit{S. aureus} disease and was a target of the natural host response to this pathogen. This immune recognition during human infection provides strong evidence that LukAB is produced \textit{in vivo}, suggesting a role in \textit{S. aureus} pathogenesis, as seen with other important staphylococcal antigens (42). Novel therapeutic and preventive options against \textit{S. aureus} are urgently needed, and recent reports suggest the benefits of a potential vaccine targeting staphylococcal exotoxins (24, 43), with the goal of preventing severe invasive disease rather than colonization or noninvasive illness (9). Given the functional antibody response produced against LukAB during invasive infection, further investigation of the role of this toxin in human disease is warranted.

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