Staphylococcus aureus infection induces protein A–mediated immune evasion in humans

Noel T. Pauli,1,2 Hwan Keun Kim,3 Fabiana Falugi,3 Min Huang,2 John Dulac,2 Carole Henry Dunand,1,2 Nai-Ying Zheng,2 Kaval Kaur,1,2 Sarah F. Andrews,1,2 Yunping Huang,2 Andrea DeDent,3 Karen M. Frank,4 Angella Charnot-Katsikas,4 Olaf Schneewind,3 and Patrick C. Wilson1,2

1Committee on Immunology; 2Department of Medicine, Section of Rheumatology, The Knapp Center for Lupus and Immunology Research; 3Department of Microbiology; and 4Department of Pathology, The University of Chicago, Chicago, IL 60637

Staphylococcus aureus bacterial infection commonly results in chronic or recurrent disease, suggesting that humoral memory responses are hampered. Understanding how S. aureus subverts the immune response is critical for the rescue of host natural humoral immunity and vaccine development. S. aureus expresses the virulence factor Protein A (SpA) on all clinical isolates, and SpA has been shown in mice to expand and ablate variable heavy 3 (VH3) idiotype B cells. The effects of SpA during natural infection, however, have not been addressed. Acutely activated B cells, or plasmablasts (PBs), were analyzed to dissect the ongoing immune response to infection through the production of monoclonal antibodies (mAbs). The B cells that were activated by infection had a highly limited response. When screened against multiple S. aureus antigens, only high-affinity binding to SpA was observed. Consistently, PBs underwent affinity maturation, but their B cell receptors demonstrated significant bias toward the VH3 idiotype. These data suggest that the superantigenic activity of SpA leads to immunodominance, limiting host responses to other S. aureus virulence factors that would be necessary for protection and memory formation.

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and surface-bound virulence factor that is present on most clinical isolates and has been shown to disrupt the humoral immune response in mice (Falugi et al., 2013). SpA contains four or five immunoglobulin-binding domains capable of binding both the Fc of IgG antibodies (preventing opsonophagocytosis) and the Fab of Variable Heavy 3 (VH3) idiotype antibodies (via a superantigen domain that binds to the complementary determining region 2 [CDR2] and Framework 1 and 3 [FRM1 and FRM3]; Björk et al., 1972; Potter et al., 1996; Graille et al., 2000). The VH3-family of immunoglobulin idiotypes represents the largest portion of VH genes in B cell populations in humans (Cook and Tomlinson, 1995). VH3 B cell receptor transgenic mice treated with SpA exhibit a VH3 B cell population expansion and ablation, suggesting a mechanism of S. aureus immune evasion by depletion of the B cell repertoire (Goodyear and Silverman, 2003). We report that in human infection, S. aureus may elude immune protection by a related but distinct mechanism of immune evasion.

To gain insight into the induction of B cell responses by S. aureus, we studied the repertoire and specificity of plasmablasts (PBs) activated by infection at the resolution of monoclonal antibodies (mAbs). Unlike plasma cells or memory B cells, PBs only exist during an infection or challenge and therefore comprise the cells of an active immune response (Wrammert et al., 2008; Smith et al., 2009). Importantly, individual PB antigen specificities are selected into long-term B cell memory (Wrammert et al., 2008; Li et al., 2012; Xu et al., 2012). It has been estimated that ~0.01% of human B cells bind a given conventional antigen during an immune response, whereas 3,000-fold more, or ~30%, are predicted to bind to SpA (Silverman et al., 1993; Wrammert et al., 2008; Li et al., 2012). We hypothesized that S. aureus evades the human immune response through the immunodominant activation of B cells by SpA binding. We observed enhanced plasmablast responses against SpA and little to no responses to 15 other S. aureus virulence factors, including those commonly found to induce serum responses (Dryla et al., 2005; Kim et al., 2010; Zeccioni and Scali, 2013; Lu et al., 2014). These responses were biased to VH3 idiotype antibodies and displayed evidence of germinal center affinity-maturation against SpA. These data suggest that SpA-mediated immune evasion occurs via the immunodominant activation of B cells in infected humans, leading to the clinical phenotype of recurrent infections. We propose that effective vaccination and clearance of chronic or recurrent infections will first require potent antibody-mediated neutralization of SpA superantigen activity.

RESULTS AND DISCUSSION

S. aureus–infected individuals respond serologically to S. aureus antigens

Peripheral blood was collected from 15 otherwise healthy individuals experiencing S. aureus infections and 3 individuals experiencing chronic or repeated S. aureus infection (Table 1). Serologically, patients exhibited reactivity to a range of S. aureus antigens similar to that observed in previous studies (Fig. 1 A; Dryla et al., 2005; Verkaik et al., 2009; Colque-Navarro et al., 2010). Verified chronic or repeated infections were represented in our cohort (n = 3, patients 055, 060, and 064); these chronically or repeatedly infected patients exhibit positive serological responses similar to the cohort as a whole, indicating that these serum antibody levels were not sufficiently protective to eliminate the secondary infections (Table 1 and Fig. 1 A). We used this cohort to investigate the mechanistic basis for poor humoral protection and memory formation during S. aureus infection.

Plasmablast response to S. aureus infection is germinal center experienced

Germinal centers (GC) are immune structures responsible for the generation of high-affinity, T cell-dependent antibody responses. Poor humoral responses toward S. aureus could be the result of limited affinity maturation of B cells in GCs, which would result in limited somatic hypermutation (SHM). We characterized the active B cell response by isolating individual PBs and generating mAbs from their B cell receptor (BCR) transcripts (Wrammert et al., 2008; Smith et al., 2009). Sequence analysis was conducted on PB BCR transcript from 10 infected individuals and mAbs were generated from seven; in total, 134 mAbs were generated (Table 1). We observed expanded PB populations in S. aureus infected subjects (n = 17, 16 total patients), similar to expansions observed during influenza infection (n = 9), that were significantly larger than in naïve healthy subjects (n = 54; Fig. 1 B; Wrammert et al., 2008, 2011). The PBs from S. aureus infections contained somatically mutated BCRs with an average of 16.95 ± 1.71 mutations, similar to what we have observed in influenza infection–generated PBs (18.54 ± 7.43; P = 0.1357; Fig. 1 C; Wrammert et al., 2008). Additionally, in all of our patients, the majority of S. aureus PBs underwent GC-mediated class-switching to IgA and IgG (Fig. 1 D). Therefore, infection–responding PBs are antigen–experienced and GC-affinity-matured B cells.

Plasmablast repertoire to S. aureus infection is VH3–biased and highly limited to Protein A reactivity

As mentioned earlier, SpA contains four or five immunoglobulin-binding domains capable of binding both the Fc portion of IgG antibodies and the Fab of VH3-idiotype antibodies via a nonspecific superantigen domain (Fig. 2 A; Forsgren, 1970; Björk et al., 1972; Potter et al., 1996; Graille et al., 2000; Goodyear and Silverman, 2003; Falugi et al., 2013). To better understand the effects of S. aureus infection on the PB BCR repertoire, we sequenced 567 individual PBs from infected individuals (n = 11 from 10 unique patients; Fig. 2 B). These sequences were compared with 338 naïve B cell sequences from healthy individuals (n = 7) and 1,373 PBs from influenza vaccinated individuals (n = 14; unpublished data; Wrammert et al., 2008, 2011). B cells containing VH3 idiotypes were preferentially activated by S. aureus infection, both in total and when averaged by patient (Fig. 2 B). Importantly, VH3-biased responses were not observed in naïve B cells from the same blood sample, showing that our cohort does not
directly assay only SpA’s superantigenic properties (Kim et al., 2010; Falugi et al., 2013). Thus, *S. aureus* infection PBs are deficient in binding to the clinically relevant virulence factors assayed, with the exception of SpA KK, indicating a preferential induction of SpA-reactive B cells in our cohort (Goodyear and Silverman, 2003; Falugi et al., 2013). As the VH3 idiotype is the largest immunoglobulin gene family in humans and has the largest representation within naive B cell populations (Cook and Tomlinson, 1995), preferential activation of nonspecific VH3 using B cells could drive immune evasion. As mentioned previously, naive B cells bind SpA 3,000-fold more frequently than a conventional antigen due to its nonspecific, superantigenic VH3-binding capacity (Silverman et al., 1993; Cook and Tomlinson, 1995; Li et al., 2012).

have a natural predisposition toward increased production of VH3 B cells; instead, this bias is a result of infection (Fig. 2 C). Because SpA is known to bind to VH3-expressing B cells, we wanted to determine if there was a biased response toward SpA at the expense of targeting antigens and virulence factors important for protection (Sasano et al., 1993; Goodyear and Silverman, 2003). We screened our 134 infection-generated mAbs by ELISA against a panel of *S. aureus* virulence factors known to be critical for host infection (Fig. 2 D; Kim et al., 2010; Falugi et al., 2013 Zecconi and Scali, 2013; Lu et al., 2014). To our surprise, although a serological response had been observed in these patients against multiple antigens, we only found reactivity toward SpA. For this we used SpAkk that is a mutant of SpA that doesn’t bind the IgG-Fc due to ablation of its binding site, which provides the opportunity to

### Table 1. *S. aureus*-infected patient cohort

| Patient number | Age | Sex | Race | Type of *S. aureus* infectiona | Treatmentb | Site of infectionc | Sample collection time point after positive culture | Chronicd or recurrente infection? | PB % of total B cells | No. of PB sequences | No. of mAbs generated |
|----------------|-----|-----|------|--------------------------------|------------|-----------------|-----------------------------------------------|----------------------------------|----------------------|---------------------|-------------------------|
| 002-10-007     | 49  | M   | W    | *S. aureus*                     | Unreported | SSTI            | 7 (laboratory infection)                       | N                                | 0.30                 | 40                  | 11                     |
| 005-10-015     | -   | F   | W    | *S. aureus*                     | Unreported | SSTI            | 7 (laboratory infection)                       | N                                | 1.70                 | 10                  | 6                      |
| 055-11-077     | 52  | F   | W    | MSSA                            | A, L       | SSTI            | 9                                             | N                                | 0.71                 | 56                  | -                      |
| 055-11-080     | 52  | F   | W    | MSSA                            | A, L       | SSTI            | 18                                            | Y                                | 1.96                 | 49                  | -                      |
| 056-11-078     | 19  | F   | W    | MRSA                            | K, M       | SSTI            | 9                                             | N                                | -                    | -                    | -                      |
| 057-11-079     | 68  | F   | -    | MRSA                            | C          | SSTI            | 4                                             | N                                | 2.50                 | -                    | -                      |
| 058-11-081     | 31  | M   | -    | MSSA                            | C, S       | SSTI            | 4                                             | N                                | 0.14                 | 53                  | 26                     |
| 060-11-083     | 57  | F   | -    | MRSA                            | C, B       | SSTI            | 8                                             | N                                | 1.12                 | 56                  | 14                     |
| 061-11-084     | 63  | F   | -    | MRSA                            | B, D, C    | SSTI            | 11                                            | Y                                | 1.47                 | -                    | -                      |
| 062-11-085f    | 34  | F   | -    | MRSA                            | S          | SSTI            | 18                                            | N                                | 0.76                 | -                    | -                      |
| 063-11-090     | 29  | F   | -    | MRSA                            | B, C       | SSTI            | 20                                            | N                                | 0.95                 | -                    | -                      |
| 064-11-092     | 18  | M   | -    | MRSA                            | B, D, C    | SSTI            | 8                                             | N                                | 1.12                 | 56                  | 14                     |
| 070-12-001     | 29  | F   | W    | MSSA                            | V, An, Ce, O | SSTI and P   | 12                                            | N                                | 0.56                 | 55                  | 19                     |
| 070-12-002     | 29  | F   | W    | MSSA                            | V, An, Ce, O | SSTI and P   | 13                                            | N                                | 0.26                 | -                    | -                      |
| 073-11-007     | 46  | M   | AA   | MSSA                            | V, Cef, D  | SF              | 17                                            | N                                | 1.05                 | 66                  | 26                     |
| 075-12-027     | 28  | F   | AA   | MSSA                            | C          | SSTI            | -                                             | N                                | -                    | -                    | -                      |
| 076-12-038     | 68  | F   | W    | MSSA                            | D, T, M    | SSTI            | 28                                            | N                                | -                    | -                    | -                      |
| 082-12-080     | 24  | M   | -    | MSSA                            | Q, Cef     | M/B >70 d      | 4.02                                          | N                                | 0.14                 | 53                  | 26                     |
| 084-13-003f    | 47  | M   | AA/W | MRSA                            | C, L, D, Cip, Cef, F, R, B | SSTI      | 3                                             | N                                | 1.43                 | 45                  | -                      |
| 099-13-102     | 22  | F   | W    | *S. aureus*                     | V          | SSTI            | -                                             | N                                | 2.74                 | 51                  | -                      |

aMRSA, methicillin-susceptible *S. aureus* (MSSA), *S. aureus* denotes *S. aureus* infections of unknown β-lactam antibiotic resistance.
bDrug key: Augmentin (A), Ancef (An), Bactrim (B), Clindamycin (C), Cefepime (Ce), Cefazolin (Cef), Ciprofloxacin (Cip), Doxycycline (D), Flagyl (F), Fluconazole (Fl), Keflex (K), Linezolid (L), Mupirocin (M), Oxacillin (O), Silvadene (S), Tetracycline (T), Vancomycin (V).
cSite of infection key: Conjunctival (C), Bacteremia (B), Muscle (M), Nasal (N), Periosteum (P), Sinus (S), Synovial Fluid (SF), Skin and Soft Tissue Infection (SSTI).
dChronic infections were infections that lasted ≥6 wk.
eRecurrent infections were indicated as *S. aureus* infections following presumed clearance of initial infection presented in this study.
fComments: Patient 062-11-085 had other gram negatives and gram positives in laboratory culture which were treated with (Cip) and Azithromycin. Patient 084-13-003 diagnosed Onychomycosis treated with (Fl).
Figure 1. Infected patient sera responds to *S. aureus* antigens and infection–induced plasmablasts exhibit germinal center affinity maturation. (A) Sera from individuals infected with *S. aureus* (n = 18, circles) and control uninfected individuals (n = 21, triangles) were tested for recognition of the indicated bacterial surface antigens by immunoblot. Statistical analysis was performed using the unpaired, two-tailed Student’s *t* test. Red lines indicate mean values. Open circles indicate patients with verified chronic or recurrent infections. (B) Plasmablasts (PBs) were identified by flow cytometry (CD19+CD3-CD27++CD38++), and percentages of PBs in total B cells from *S. aureus*–infected patients (n = 17) were determined. Red (055–11077) and blue (055–11080) data points represent two blood draws from the same patient on different days of a chronic infection. Results were compared with historical data from the 2009 pandemic H1N1 influenza infection (n = 9) and naive healthy patients (n = 54). Statistical analysis was performed using the Mann-Whitney test. Red lines represent mean values. (C) Peripheral blood cells were isolated from patients with *S. aureus* or influenza infections as well as from individuals receiving influenza vaccines. The number of somatic hypermutations was determined in *S. aureus* (n = 7) or influenza infected (n = 6) PBs, vaccination–induced memory B cells and germinal center (GC) B cells (n = 15), and naive B cells (n = 7). Each point represents one individual. Statistical analysis was performed using the Mann-Whitney test for significance. Red lines indicate median values. (D) PBs were isolated from *S. aureus*–infected patients, and the immunoglobulin isotypes expressed by these PBs were assessed by sequence analysis. Numbers on x axis refer to individual patient designations, and the n value refers to the number of PBs isolated from each patient. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.
Figure 2. *S. aureus* infection–induced plasmablasts are VH3–biased and respond to SpA but lack reactivity to other *S. aureus* antigens.

(A) Illustration of wild-type and mutated SpA protein. (B) PBs were isolated from patients infected with *S. aureus* (n = 10) or influenza (n = 14), or from uninfected controls (n = 7). The percentage of cells containing VH3 idotypes was determined in naive B cells or responding PBs by sequencing BCR genes. Each point represents a single patient. Statistical analysis was performed using the Mann-Whitney test. Red (055–11077) and blue data (055–11080) points represent two blood draws from the same subject on different days. Statistics were calculated from the mean value of these two data points. Eleven total blood samples were analyzed from the ten patients. Red lines represent mean value. Pie charts represent the overall percentage of VH3 versus...
**S. aureus** infection–generated plasmablasts are affinity-matured toward Protein A

Despite the nonspecific nature of the superantigen–mediated VH3–SpA interaction, one would predict that this binding would still direct B cells to undergo affinity maturation to SpA, increasing the affinity of the antibodies for SpA. We first determined whether mAbs from **S. aureus** infection–induced PBs bind with higher avidity to SpAkk than mAbs from influenza-specific plasmablasts. Of these, 53% were VH3 family members (unpublished data; Wrammert et al., 2008, 2011). Total influenza-specific antibodies bound SpAkk at a frequency of 29%, verifying previous human data demonstrating that ~30% of all B cells bind SpA nonspecifically via superantigen–mediated interaction with VH3-encoded Fab regions (Silverman et al., 1993; Fig. 3 A). The frequency of mAbs bound by SpA was increased (48%) in the **S. aureus**–infected cohort (Fig. 3 A); **S. aureus**–infected individuals produced PBs with greater relative avidity to SpAkk compared with our influenza-vaccinated control cohort on both a per-patient basis and as a population (Fig. 3 A). Also, we observed that IgG isotype antibodies had a greater frequency of binding and modestly enhanced avidity to SpAkk when compared with IgA isotype antibodies (unpublished data). Enhanced binding was only detected in **S. aureus** infection VH3 PBs (Fig. 3 B), indicating that the binding was superantigen mediated. To assay the relevance of mutations toward SpA binding, we reverted five mAbs, four VH3 and one non-VH3, back to their germline configuration and analyzed their binding to SpAkk or SpAkka (Fig. 3 C). SpAkka, or nontoxicogenic SpA, is a mutant of SpA lacking superantigenic-Fab and IgG-Fc binding; antibodies bind it via specific antibody–antigen interaction rather than nonspecific superantigen interaction (Fig. 2 A; Kim et al., 2010). Reversion did not change the binding patterns of three of our VH3 mAbs, 070 2A02, 002 1C06, and 061 1G04, to SpAkk (Fig. 3 C, top). This is expected because superantigenic binding is independent of antigen–binding regions such as CDR3 (Potter et al., 1996). One VH3 mAb, 073 2F03, gained binding to SpAkk after reversion of its mutations, indicating that the mutations were partially disrupting superantigenic binding. Interestingly, a VH4-59 mAb that would not be predicted to bind SpAkk, 060 2A03, lost binding upon germline reversion, suggesting this B cell was activated against SpA in a superantigen–independent fashion. Three antibodies bound SpAkka (073 2F03, 061 1G04, and 060 2A03), indicating that they bound epitopes outside of the superantigen-binding site (Fig. 3 C, bottom). Importantly, these three mAbs lost the ability to bind to SpAkka upon mutation reversion, suggesting that the selected mutations mediated additional interactions of the antibody outside of the SpA superantigen-binding site (Fig. 3 C, bottom). Collectively, these data demonstrate that the PB response during infection is selected by and affinity-matured toward SpA.

Plasmablasts represent a readout of what the immune response actively targets during infection and correlates with memory B cell specificity (Wrammert et al., 2008, 2011; Smith et al., 2009; Li et al., 2012; Smith et al., 2013). However, in **S. aureus** infection, PBs specific to virulence factors other than SpA were undetectable, indicating that the cells inducing the serological response we detected must be quite rare. Our observation was corroborated by Excelimmune, Inc., during their efforts to generate **S. aureus**–specific antibodies. From 15 infected individuals, they found only 90 out of 6,877 plasmablasts screened bound to non-SpA **S. aureus** antigens, whole-killed bacteria, or bacterial supernatant. On an individual donor basis, plasmablast reactivity to **S. aureus** antigens was also low, with an observed binding frequency between 0–3% (personal communication, Excelimmune, Inc.). We propose that SpA dominates the germinal center response, preventing the production of specific and effective antibody responses to other staphylococcal antigens.

SpA binds 30% of total B cells; this is in stark contrast to conventional antigens, which only bind to 0.01% (Silverman et al., 1993). Affinity maturation is tied directly to the quantity of antigen captured and processed, likely making SpA the immunodominant antigen presented by B cells to T follicular helper cells (Tfh) cells, restricting affinity maturation predominately to SpA (Silverman et al., 1993; Gitlin et al., 2014). This model is supported by our findings: 48% of activated PBs bind to SpA, bind with heightened avidity, and accumulate
Figure 3. *S. aureus* infection–induced plasmablasts have undergone SpA affinity maturation. (A) mAbs were generated from *S. aureus*–infected patients (*n* = 7) or healthy controls (*n* = 24) as in Fig. 2D, and binding to SpAKK was assessed by ELISA. Data were presented as the mean binding per antibody (left; each point represents one antibody) or the averaged per subject (right; each point represents one patient). 134 and 160 mAbs were generated from *S. aureus*–infected individuals (6–32 mAbs per person) and controls (2–22 mAbs per person), respectively. Diagram (left) illustrates the area under the curve (AUC), which accounts for both maximum binding absorbance and half-maximum binding (KD). Blue and red dots on AUC graph (right) correspond to same color on the AUC diagram. AUC statistical analysis was performed using the Mann-Whitney test. Results shown are the mean of three independent replicate experiments. Pie charts represent the frequency of mAbs binding to SpAKK (red portion) as determined by two standard deviations above the mean binding of VH3~−~ influenza B cells (1.15 X 10^−7), indicated by blue dotted line. This was compared with SpAKK nonbinding mAbs (tan). Antibodies falling above the blue dotted line were scored as being positive to SpAKK binding, falling below the line the antibodies would be scored as negative. Pie chart statistical analysis was performed using the χ^2~ test~. (B) VH3~−~ and VH3~+~ mAbs specific for influenza (control) or *S. aureus* were tested for binding to SpAKK by ELISA and presented as AUC (Control VH3~−~ and VH3~+~ mAbs: *n* = 75 and 85, respectively; *S. aureus* VH3~−~ and VH3~+~ mAbs: *n* = 33 and 101, respectively). Pie charts show the frequency of mAbs that bind to SpAKK or do not bind as determined in A. Statistical analysis performed as determined in A. Results shown are the mean of three independent replicate experiments. (C) Infection-generated antibodies were compared with their native germline sequences, mutations that were outside the junctional regions were removed. These reverted antibody sequences were then cloned and expressed in HEK293 cells. Binding of reverted mAbs to (C top) SpAKK or (C bottom) SpAKKAA was determined by ELISA and presented as AUC. Black lines indicate VH3~+~ PBs and red lines represent VH3~−~ PBs. "I" indicates infection generated mAbs; "R" represents germline reversion mAbs. The top number on the x axis is the patient designation, and bottom labels refer to individual mAb clones. *, *P* ≤ 0.05; ***, *P* ≤ 0.001.
mutations in their variable genes that mediate superantigen-independent interactions. Whereas in mice, as previously reported, SpA induces ablation of VH3 B cells, we observe a SpA-reactive, VH3-biased response repertoire in patients during natural infection (Table 1; Goodyear and Silverman, 2003). Recent work by Falugi et al. (2013) corroborates part of our findings in a mouse model, demonstrating that SpA is able to suppress effective serological responses during infection and is critical for bacterial immune evasion. We propose that nonspecific expansion and activation of PBs by SpA allows S. aureus to prevent protective humoral immune responses and the generation of sufficient memory to avoid future infections. Therefore, we believe that the neutralization of SpA function through vaccination with recently generated candidates, such as nontoxicogenic SpA, will be critical for the creation of a successful, multiantigen S. aureus vaccine (Kim et al., 2010).

MATERIALS AND METHODS

Patient sample collection. This study was approved and blood was collected in accordance with the University of Chicago Institutional Review Board (IRB #09-043-A). All patients provided informed consent to the study. Relevant patient information has been disclosed in Table 1. Patients who had other concomitant infections/autoimmune disease or were on immunosuppressive regimens were excluded from this study. Chronic infections were defined as patients with infections lasting >6 wk. Recurrent infections were defined as infections returning after presumed clearance or clinical resolution of initial infection.

Flow cytometry and single-cell sorting. Peripheral blood B cells were enriched from whole blood using RosetteSep Human B Cell Enrichment Cocktail (STEMCELL Technologies). Plasmablasts were sorted using a FACSAria II (BD) in 0.2% BSA/PBS. Plasmablasts were identified as CD19+CD3-CD27+CD38+ and naive B cells were identified as CD19+CD3-CD27-CD38- as described previously (Smith et al., 2009). CD19, CD27, and CD38 were defined as infections returning after presumed clearance or clinical resolution of initial infection.

Monoclonal antibody generation and sequence analysis. mAbs were generated from plasmablasts as previously described (Wrammert et al., 2008; Smith et al., 2009). In brief, single-cell reverse transcription and PCR were used to amplify the heavy and light chain variable genes. Single-cell cDNA was analyzed using the international ImMunoGeneTics (IMGT) information database and JOINSSolver. The genes were cloned into human IgG1 and kappa or lambda expression vectors and transiently transfected into 293A cell lines for expression. Sequence information is available at GenBank under accession nos. KM603669-KM604222.

Enzyme-linked immunosorbent assays. Recombinant protein antigen was coated at a concentration of 2 μg/ml overnight at 4°C in carbonate-binding buffer. Eight 1:4 serial dilutions of antibody were prepared from a starting concentration of 10 μg/ml. Intermediate washes were performed with PBS/0.05% Tween. Plates were blocked for 1 h at 37°C with 2% FBS in PBS, and then incubated with horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) for 1 h at 37°C to detect binding. Development was performed using Super AqueBlue ELISA Substrate (eBioscience) at an absorbance of OD405.

Statistical analysis. The unpaired two-tailed Student's t test was used to analyze the significance of serological assays. The χ² test was used to analyze the significance of naïve B cell versus PB VH3 usage and frequency of mAbs to bind SpA. The two-tailed Mann-Whitney test was used to analyze the significance of monoclonal ELISAs, flow cytometry, mutational frequency, and VH3 skewing averaged by patient. Statistical analysis was performed using GraphPad Prism 5 software.

Mutation reversions. Heavy and light chain sequences were compared with germline sequences using the IMGT information database. Mutations detected within the sequenced V genes were reverted back to their database germline configuration. The genes were synthesized by GenScript USA Inc., followed by expression as described under monoclonal antibody generation (Smith et al., 2009). Junctional mutations were not reverted because we were unable to confirm whether these occurred during somatic rearrangement or as a result of somatic hypermutation.

Serological analysis. Affinity-purified proteins were spotted onto nitrocellulose membrane (GE Osomonic) at 1 μg/μl blocked with 5% milk and incubated with 2 μl human sera samples. Membranes were washed and incubated with secondary goat anti-human IgG antibody at 1:10,000 (LI-COR) and near-infrared fluorescence signal intensities quantified using Odyssey (LI-COR).

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