Immune evasion by a staphylococcal inhibitor of myeloperoxidase

Nienke W. M. de Jong[a], Kasra X. Ramyar[b], Fermin E. Guerra[c], Reindert Nijland[a,d], Cindy Fevre[a], Jovanka M. Voyich[c], Alex J. McCarthy[a], Brandon L. Garcia[b], Kok P. M. van Kessel[a], Jos A. G. van Strijp[a,1], Brian V. Geisbrecht[b,2], and Pieter-Jan A. Haas[a,2]

[a]Medical Microbiology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands; [b]Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS 66506; [c]Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717; and [d]Laboratory of Phytopathology, Wageningen University, 6708 PB Wageningen, The Netherlands

Edited by Richard P. Novick, New York University School of Medicine, New York, NY, and approved July 18, 2017 (received for review April 28, 2017)

Staphylococcus aureus **is highly adapted to its host and has evolved many strategies to resist opsonization and phagocytosis. Even after uptake by neutrophils, *S. aureus* shows resistance to killing, which suggests the presence of phagosomal immune evasion molecules. With the aid of secretome phage display, we identified a highly conserved protein that specifically binds and inhibits human myeloperoxidase (MPO), a major player in the oxidative defense of neutrophils. We have named this protein "staphylococcal peroxidase inhibitor" (SPIN). To gain insight into inhibition of MPO by SPIN, we solved the cocrystal structure of SPIN bound to a recombinant form of human MPO at 2.4-Å resolution. This structure reveals that SPIN acts as a molecular plug that prevents H_2O_2 substrate access to the MPO active site. In subsequent experiments, we observed that SPIN expression increases inside the neutrophil phagosome, where MPO is located, compared with outside the neutrophil. Moreover, bacteria with a deleted gene encoding SPIN showed decreased survival compared with WT bacteria after phagocytosis by neutrophils. Taken together, our results demonstrate that *S. aureus* secretes a unique proteinaceous MPO inhibitor to enhance survival by interfering with MPO-mediated killing.**

immune evasion | myeloperoxidase | neutrophil | phagocytosis | *Staphylococcus aureus*

The bacterium *Staphylococcus aureus* is a rising threat to human health. Thirty percent of healthy adults are colonized with this bacterium, resulting in an increased risk for infections ranging from abscesses to endocarditis (1). Neutrophils play a prominent role in fighting staphylococcal infections (2), as their intracellular granules contain numerous antimicrobial proteins and components for generating bactericidal reactive oxygen species (ROS). After *S. aureus* is phagocytosed, neutrophils' azurophilic granules fuse with the phagosome and release their contents (3). The five essential components of NADPH oxidase then assemble in the phagosomal membrane and become active (4). Active NADPH oxidase produces superoxide from O_2, which converts to hydrogen peroxide (H_2O_2) either spontaneously or by the action of superoxide dismutase. Myeloperoxidase (MPO) catalyses the reaction of H_2O_2 with chloride to generate hypochlorous acid (HOCl), which is a major effector in the oxidative defense of neutrophils (5). MPO also forms radicals by oxidizing a wide range of substrates, such as tyrosine, nitrite, nitric oxide, and phenols (6).

While the pathogen is taken up rapidly by phagocytes, mainly neutrophils and macrophages, not all bacteria are killed and these phagocytes can therefore act as so called "Trojan Horses" and distribute a pathogen away from the initial site of infection (7). To counteract the manifold antimicrobial defenses of neutrophils, *S. aureus* has evolved specific evasion molecules to inhibit intracellular killing (8). For example, the golden pigment staphyloxanthin serves as an antioxidant and is known to protect *S. aureus* against ROS (9). Catalase is yet another enzyme important for resistance against oxidative stress. This enzyme converts H_2O_2 into H_2O and O_2 and is considered to be a virulence factor. *S. aureus* also expresses an alkyl hydroperoxide reductase (*ahpC*) that contributes catalase-like activity. Whereas AhpC is believed to detoxify endogenously produced hydrogen peroxide, catalase appears more important for protection against external oxidative stress (10). Finally, *S. aureus* produces specific evasion proteins that disrupt phagosomal membranes, such as phenol-soluble modulins, hemolysin-α, and leukocidin AB (8). Together, these evasion molecules are believed to contribute to bacterial survival following phagocytosis.

Proteomic studies have shown that between 100 and 200 proteins are secreted from *S. aureus*, many with unknown functions (11). Consequently, known evasion molecules are likely to represent only a small fraction of the total repertoire. Therefore, we recently developed a phage display approach (12) as a nonbiased high-throughput method to screen for new potential staphylococcal immune evasion molecules. Since *S. aureus* can survive within the phagosome, but also because recent work suggests that SaeR/S regulated factors exist that inhibit neutrophil ROS production (13), we screened this staphylococcal phage library against several intracellular proteins of neutrophils.

Through this approach, we identified the hypothetical protein NWMN_0402 as an evasion factor. We have named this protein "staphylococcal peroxidase inhibitor" (SPIN), as it is able to bind and inhibit MPO. Here, we characterize SPIN and detail the structural basis for MPO inhibition by SPIN. We further show that the production of SPIN is up-regulated after phagocytosis of

Significance

Staphylococcus aureus **secretes numerous proteins to evade our innate immune system, for example to evade opsonization and phagocytosis by neutrophils. Here we describe the discovery that *S. aureus* has evolved a protein, called SPIN, that specifically binds and inhibits the human myeloperoxidase enzyme (MPO). MPO is located inside the granules of neutrophils and is important in the oxidative burst against pathogens. We identify the molecular mode of action of SPIN inhibiting MPO, illustrate this with the cocrystal structure, and show that SPIN is important for bacterial survival by MPO-dependent killing. Our study shows that *S. aureus* fights back after it is engulfed by neutrophils, which will help our understanding of the complex nature of *S. aureus* infections.**

Author contributions: R.N., C.F., J.M.V., B.L.G., K.P.M.v.K., J.A.G.v.S., B.V.G., and P.J.A.H. designed research; N.W.M.d.J., K.X.R., F.E.G., A.J.M., B.L.G., and B.V.G. performed research; C.F., K.P.M.v.K., and P.J.A.H. were involved in the discovery of the protein and its function; and N.W.M.d.J., K.X.R., K.P.M.v.K., J.A.G.v.S., B.V.G., and P.J.A.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 5UZU).

[1]To whom correspondence should be addressed. Email: j.vanstrijp@umcutrecht.nl.

[2]B.V.G. and P.J.A.H. contributed equally to this work.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1707032114/-/DCSupplemental.

S. aureus by human neutrophils and that SPIN protects *S. aureus* from MPO-mediated killing.

Results

Identification of SPIN. We designed a secretome phage display strategy to screen for unidentified evasion molecules that target neutrophil granule proteins, as described previously (12, 14). Isolated DNA of *S. aureus* strain Newman was randomly sheared and the resulting fragments were cloned in the pDJ01 phagemid vector for specific display of secretome proteins. Phages were produced upon addition of the VCSM13 helper phage. Screening was performed against total degranulate from TNF-primed, formylmethionine-leucyl-phenylalanine–stimulated neutrophils. After four rounds of selection, 48 clones were sequenced. The major hit was enriched in 36 clones and corresponded to the hypothetical ORF NWMN_0402 (accession no. BAF66674 strain Newman) (Fig. S1*A*). We named this protein SPIN due to its ability to inhibit MPO, as described in *SPIN Binds and Inhibits MPO*.

The gene encoding SPIN, denoted as *spn*, is located on genomic island α, also known as νSaα. This island is found in all *S. aureus* genomes and contains a cluster of genes encoding known evasion molecules, most notably the staphylococcal superantigen-like (*ssl*) proteins (8). *spn* is located downstream of the *ssl* cluster with the coding sequence oriented in the reverse direction (Fig. S1*B*). The genes in νSaα are often variable and each *S. aureus* lineage has a unique combination of gene variants (15). However, *spn* is located in the conserved region of νSaα (Fig. S1*C*). It was present in 83 of 84 clinical strains isolated from intensive care unit patients as well as in all completed *S. aureus* genomes included in our analysis (15).

The amino acid sequence of SPIN is highly conserved among almost all *S. aureus* clonal lineages, both human- and animal-associated strains, with 92.4% of residues conserved (Fig. S1*D*). Only two strains of clonal complex (CC)5 lineage, Mu3 and Mu50, encoded a truncated version of SPIN, lacking 35 amino acids at the C terminus. To determine if SPIN is produced in vivo during colonization or infection of the human host, sera from 20 healthy individuals were tested for the presence of antibodies against SPIN. These sera showed substantial amounts of antibody, and elicited values comparable to CHIPS (chemotaxis inhibitory protein of *S. aureus*) (Fig. S1*E*). CHIPS is a known *S. aureus* immune evasion molecule (16) that yielded a mean fluorescence intensity value in a previous multiplex assay indicative of high IgG levels, whereas the superantigens (e.g., SEI) did not (17). Because SPIN is highly conserved in *S. aureus* strains, has a high prevalence, and is expressed in vivo, we further investigated this protein as a potential evasion molecule acting on one of the granular proteins of neutrophils.

SPIN Binds and Inhibits MPO. Since we identified SPIN as a potential evasion protein, recombinant SPIN was coupled to CNBr-activated Sepharose beads and used for affinity chromatography to probe neutrophil degranulate for its binding partner. Eluted proteins were visualized by silver-stained PAGE and identified by mass spectrometry. Although elastase, proteinase 3, cathepsin G, azurocidin, lactoferrin, and MPO were all identified as potential binding partners, a subsequent SPIN capture ELISA against these potential targets only showed evidence for SPIN binding to MPO (Fig. 1*A*). Next, we used conventional surface plasmon resonance (SPR) (Fig. 1*B*) as well as AlphaScreen equilibrium competitive binding assays (Fig. S2) to validate our ELISA result. Both SPR and AlphaScreen were consistent with low nanomolar affinity binding between SPIN and MPO, and yielded comparable K_D values of 10 ± 0.6 nM and 2.0 ± 0.07 nM, respectively.

Since SPIN forms a high-affinity complex with MPO, we hypothesized that SPIN disrupts MPO function. Indeed, a colorimetric MPO activity assay demonstrated that SPIN inhibits MPO in a dose-dependent manner (Fig. 1*C*) with an apparent IC_{50} of ∼7 nM. Subsequently, we investigated whether SPIN was secreted as a functional protein in staphylococcal culture supernatant. Both

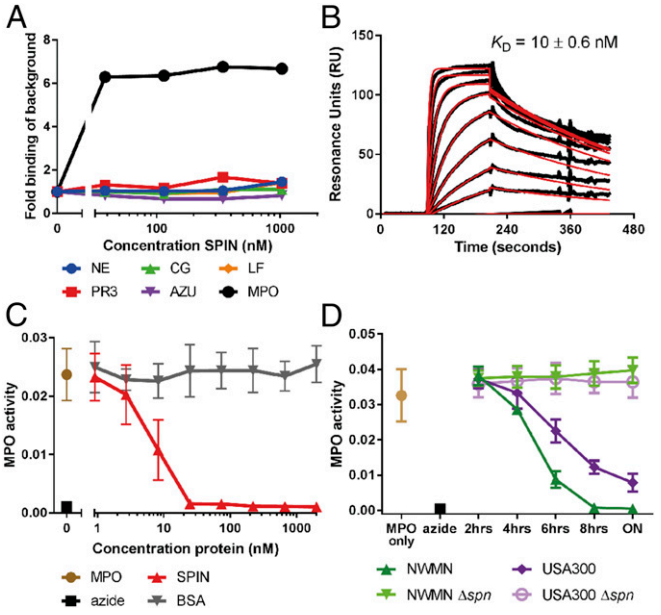

Fig. 1. SPIN binds and inhibits MPO. (*A*) An ELISA-type binding assay of C-His SPIN to all candidate proteins. AZU, azurocidin; CG, cathepsin G; LF, lactoferrin; MPO, myeloperoxidase; NE, neutrophil elastase; PR3, proteinase 3. (*B*) Characterization of SPIN binding to MPO by SPR, where SPIN was injected over a surface of native MPO. (*C* and *D*) Recombinant SPIN and *S. aureus* supernatant inhibit MPO in a dose-dependent manner. ON is overnight culture. Bars express SD with $n = 3$ for *B*–*D*.

WT strains Newman and USA300 accumulated increasing levels of MPO inhibitory activity in conditioned culture medium over time. Moreover, we also found that strain Newman supernatant showed a higher degree of inhibition compared with USA300. To determine whether this MPO inhibitory activity was caused by the SPIN protein, we generated isogenic KOs of the SPIN gene in both Newman and USA300 backgrounds. Conditioned culture medium from both KOs was completely devoid of MPO inhibitory activity (Fig. 1*D*).

We also performed a titration analysis on samples of conditioned culture supernatant from WT *S. aureus* strains Newman and USA300 (Fig. S3 *A* and *B*). The IC_{50} of these samples was 0.12% and 0.30% supernatant for Newman and USA300, respectively. By comparing this to the IC_{50} from recombinant SPIN (Fig. 1*C*), the amount of SPIN present in the overnight supernatant could be estimated. This appeared to be 5.9 μM for Newman and 2.4 μM for USA300. Interestingly, the supernatant from the Mu50 strain, which contains a truncated version of *spn* (Fig. S1*D*), showed no detectable MPO inhibitory activity in its supernatant (Fig. S3*C*), indicating a nonfunctional SPIN-variant. The slight inhibitory effect observed at low dilutions of the conditioned media is due to the inhibitory color of Todd Hewitt Broth (THB) itself (Fig. S3*C*).

Structural Basis for MPO Inhibition by SPIN. SPIN shares no significant sequence homology to other characterized proteins. Thus, we sought structural information to better understand the physical basis for SPIN binding to and inhibition of MPO. We succeeded in crystallizing a complex of SPIN bound to a recombinant form of MPO, collected X-ray diffraction data to 2.4-Å Bragg-limiting resolution, and solved the structure by molecular replacement (Table S1). Electron density maps calculated after the initial placement of a model for human MPO revealed a strong, contiguous feature that could not be explained by the presence of MPO (Fig. 2*A*). A model of the SPIN polypeptide was completed through a combination of automated chain tracing and manual building, and the structure of the complex was refined to final R_{work}/R_{free} values of 18.4% and 24.1%, respectively (Table S1). The SPIN

protein consists of the common α-helix bundle fold found in other staphylococcal evasion proteins, with a unique addition of a prominent β-hairpin at its N terminus (Fig. 2*B*). As there are no biochemical features that would promote stability of this β-hairpin in the absence of ligand (e.g., a disulfide bond), we suspect that this region of SPIN is disordered in the unbound state.

The interaction between SPIN and MPO buries 1,633 Å^2 from the bacterial inhibitor and is comprised of two conceptually distinct interfaces (Fig. 2*C*). The first interface lies at the side of the peroxidase active site entrance, and accounts for 726 Å^2 (~45%) buried in the complex. Sixteen of the 20 SPIN residues associated with this site are found on either the first or second helix of the inhibitor. The sidechains of five such residues form either hydrogen bonds or salt-bridges with groups donated by MPO, and likely help impart specificity for this enzyme. The second interface accounts for 907 Å^2 (~55%) of the area buried in the complex, and is derived exclusively from residues within the SPIN β-hairpin. All positions in this region of SPIN form extensive contacts with residues that line the MPO active site channel. We find the Gly residue in the β-hairpin turn particularly noteworthy, since it not only allows the second strand to complete the hairpin but also dictates the extent to which this hairpin can penetrate the MPO active site. The overall effect of this structural feature allows SPIN to act as a molecular plug and thereby prevent access of the H_2O_2 substrate to the reactive heme located deep within the MPO active site channel (Fig. 2*D*).

Production of SPIN by *S. aureus* Is Up-Regulated After Phagocytosis by Neutrophils. To better understand the biological context of SPIN production by *S. aureus*, we compared SPIN gene expression and promoter activity in bacteria that had been phagocytosed and not phagocytosed by neutrophils. We used strain USA300, since SPIN is constitutively expressed at low levels by strain Newman. SPIN gene expression over time was monitored using qPCR, either with or without phagocytosis. We observed a highly significant, eightfold increase in *spn* expression following phagocytosis compared with samples without neutrophils (Fig. 3*A*). This outcome is comparable to our earlier microarray study, where enhanced *spn* expression was detected after exposure to azurophilic granule proteins (18). Importantly, we did not detect a similar change in *spn* expression when we used bacteria lacking SaeR/S, which regulates production of multiple evasion factors (19). As a control, we found no significant change in *agrA* expression under any of these conditions (20) (Fig. S4).

Next, we used GFP promoter-reporter USA300 bacteria in a fibrin-thrombin matrix gel to immobilize cells. Time-lapse imaging of the bacteria revealed up-regulation of the *spn* promoter-driven GFP signal following phagocytosis by neutrophils (arrows, Fig. 3*B*). In contrast, the *spn* promoter appeared inactive in those bacteria that had not been phagocytosed (circles, Fig. 3*B*); this observation correlates with *spn* promoter activity for THB-grown bacteria, which showed activity only after 2 h of growth (Fig. S5). We further quantified the total GFP

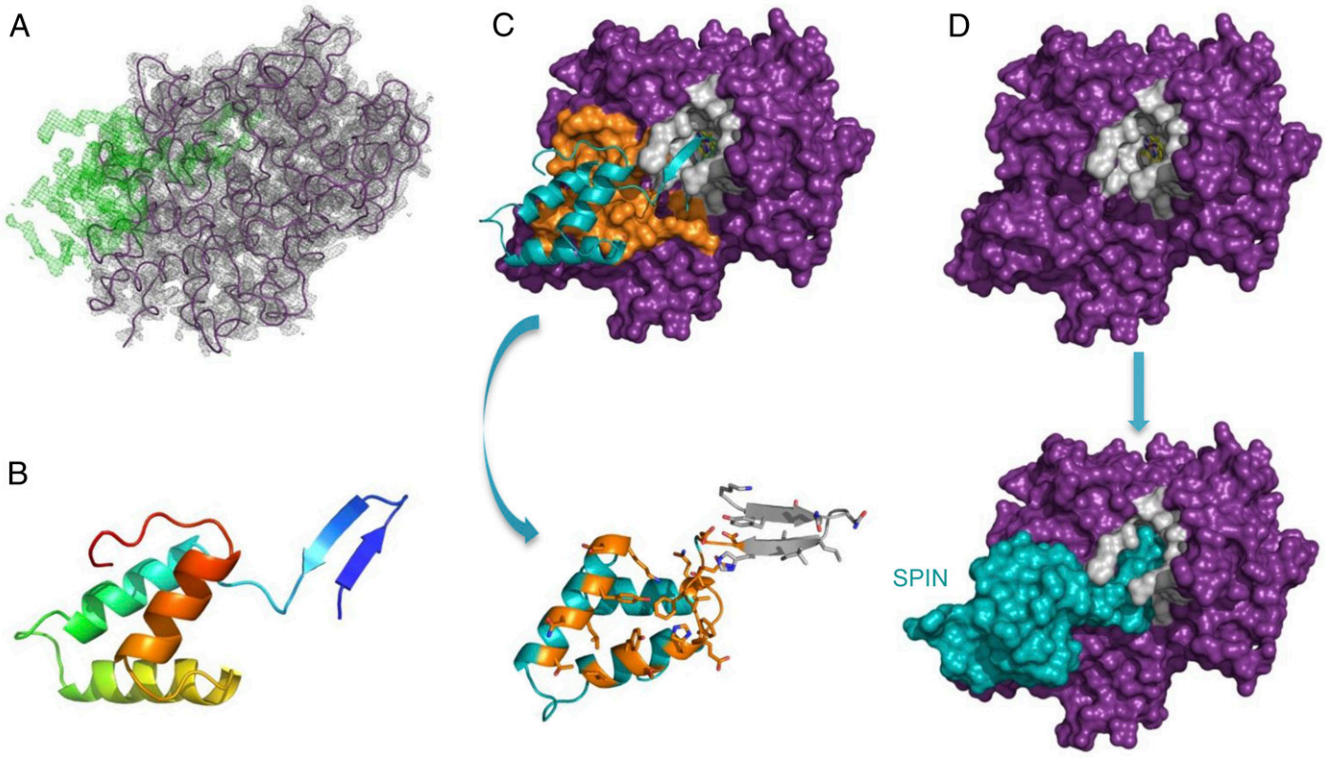

Fig. 2. Structural basis for inhibition of MPO by SPIN. (*A*) Electron density maps (2.4-Å resolution) calculated after initial placement of an MPO model (R_{free} = 28%). $2F_o$–F_c density contoured at 1.5 σ (gray cage) is shown for the MPO model (purple wire), and F_o–F_c density contoured at 3.0 σ (green cage) is attributable to SPIN. (*B*) Structure of the SPIN polypeptide depicted as a ribbon diagram. N terminus of the protein is indicated in indigo, the C terminus in red. The orientation of SPIN has been maintained across *A* and *B* for clarity. (*C*) Representation of the final model for the SPIN/MPO complex. (*Upper*) SPIN is shown as a cyan ribbon while MPO is depicted as a molecular surface. Residues comprising the first SPIN binding interface are colored orange, and residues lining the MPO active site channel are colored gray. (*Lower*) SPIN is drawn with the residues found at the first interface colored orange, and residues interacting with the MPO active site channel are colored gray. The sidechains of interfacing residues are depicted in ball-and-stick convention. Note the orientation of SPIN in the *Lower* panel is flipped 180° in the viewing plane relative to the *Upper* panel. (*D*) Surface representations provide insight into the physical basis for MPO inhibition by SPIN. (*Upper*) MPO is shown as a molecular surface with the residues lining the active site channel in gray. (*Lower*) SPIN is drawn as a cyan molecular surface according to its position in the final model of the SPIN/MPO complex. The location of the reactive site heme from native human MPO (33) is shown as a colored ball-and-stick. Note that the SPIN β-hairpin appears to completely occlude access of small molecules to the reactive site heme.

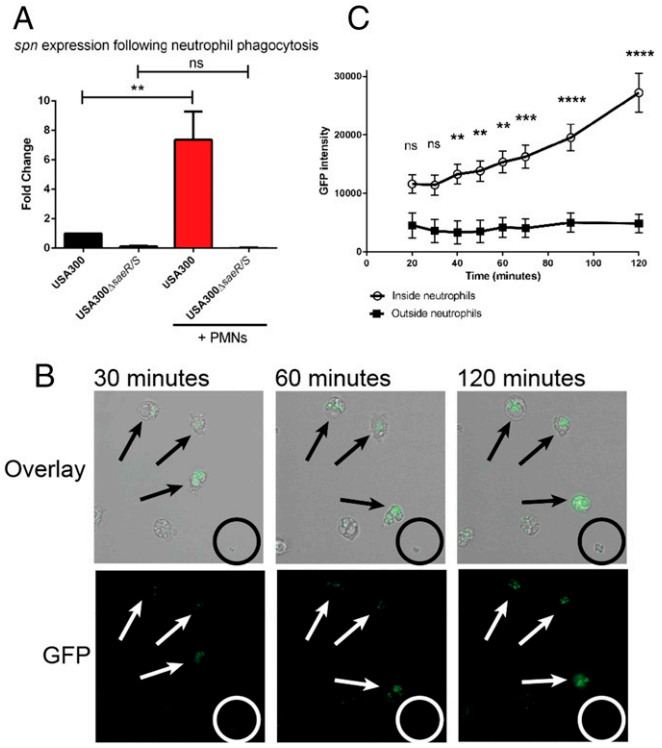

Fig. 3. Production of SPIN is up-regulated after phagocytosis inside neutrophils. (*A*) Analysis of *spn* expression following exposure to human neutrophils. Bars express SD with $n = 3$. Statistical significance was determined using one-way ANOVA. (*B*) Time-lapse analysis of SPIN expression shown as GFP fluorescence promoter-reporter USA300 in a fibrin-thrombin matrix gel. An overlay of bright-field and GFP (*Upper*) and GFP alone (*Lower*) is shown. (Magnification: 40×.) The time indicates the duration after start of phagocytosis. Arrows indicate bacteria inside neutrophils and the circle indicates a colony of bacteria growing outside neutrophils. One representative experiment is shown from three independent experiments. (*C*) Quantification of total GFP signal from neutrophil-resident or free bacteria. Three different experiments with 44 neutrophils and 26 colonies growing outside neutrophils were analyzed. Bars express SEM. Significance was determined by two-way ANOVA with Bonferroni posttest correction for multiple comparison. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$; ns, not significant.

signal for either free or neutrophil-resident bacteria for 2 h, beginning at 20 min. We found that GFP intensity increased more than twofold over time for phagocytosed, but not free-living bacteria (Fig. 3*C*).

Production of SPIN Promotes Survival Against Neutrophil-Derived ROS. Since SPIN inhibits MPO and is up-regulated following phagocytosis, we next investigated the relevance of SPIN to *S. aureus* evasion of neutrophil killing mechanisms. First we used an artificial system that mimics phagosomal ROS production using glucose oxidase (GO), which forms H_2O_2 in the presence of glucose. We evaluated bacterial survival after the addition of MPO and NaCl, which generates the bactericidal reagent HOCl (Fig. 4 *A* and *B*). Excessive quantities of GO result in bactericidal concentrations of H_2O_2, which makes it impossible to evaluate MPO-mediated killing. In limiting concentrations of GO, increasing MPO concentrations led to killing of *S. aureus* Newman (Fig. 4*A*, *Left*) and USA300 (Fig. 4*A*, *Right*). Addition of SPIN restored the survival of bacteria (Fig. 4*A*). Moreover, adding increasing concentrations of SPIN (Fig. 4*B*) resulted in a dose-dependent rescue from HOCl killing for both Newman and USA300. This demonstrates clearly that the presence of SPIN is important for protection from MPO-generated HOCl.

SPIN is neither able to bind nor inhibit mouse, horse, cow, or rabbit MPOs, hampering the development of an in vivo animal model system (Fig. S6). We therefore set out to investigate how the presence or absence of the SPIN gene influences *S. aureus* survival following phagocytosis by human neutrophils. First, the contribution of MPO to staphylococcal killing by neutrophils was investigated using the membrane-permeable MPO inhibitor AZM198 (21). Incubation of intact neutrophils with this inhibitor before washing and lysis was equally as effective as direct treatment of lysates, indicating that AZM198 is membrane-permeable and completely inhibits MPO (Fig. S7). We quantified bacterial killing by neutrophils with and without AZM198 pretreatment, and observed a difference of 26% between neutrophils with active and inactive MPO. This illustrates the modest extent of MPO-dependent staphylococcal killing by neutrophils in our experiments (Fig. 4*C*).

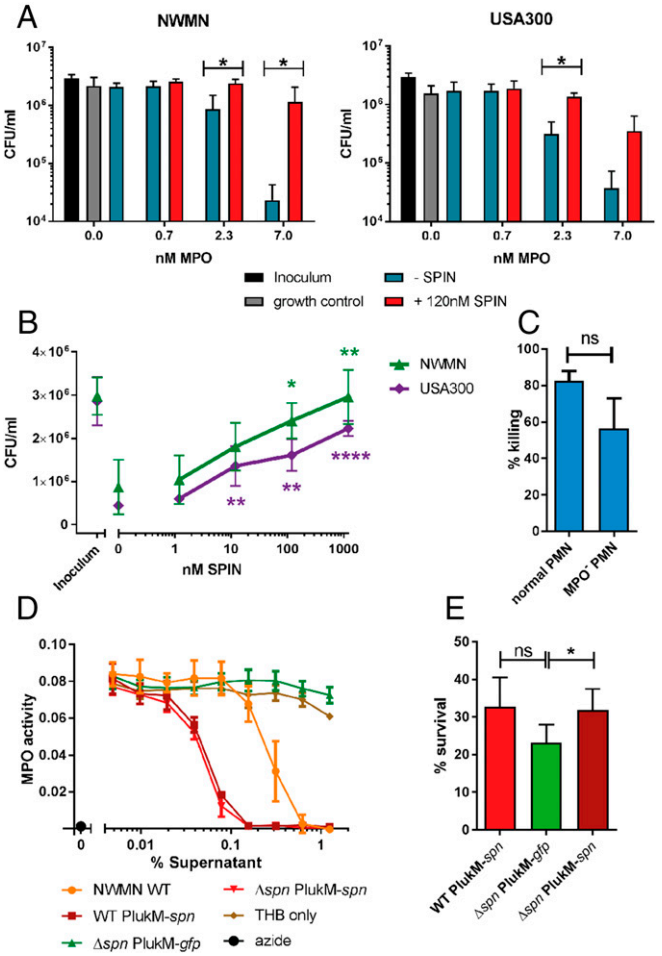

Fig. 4. SPIN is important for evasion of MPO-dependent neutrophil killing. (*A*) *S. aureus* strains Newman (*Left*) and USA300 (*Right*) were killed in a dose-dependent manner by adding 0.7–7.0 nM MPO in a coupled glucose oxidase-MPO system. Addition of 120 nM SPIN prevented ROS killing. Statistical significance was determined by two-way ANOVA with Bonferroni posttest correction for multiple comparison. (*B*) SPIN provides dose-dependent protection from HOCl-mediated killing for strains Newman and USA300 relative to 0 nM SPIN. Statistical significance was determined by one-way ANOVA with Bonferroni posttest correction for multiple comparison. (*C*) Differential bacterial survival of Newman WT from neutrophils with active or membrane-permeable AZM198-inactivated MPO. (*D*) Supernatants of overnight culture of strains WT PlukM-*spn*, Δ*spn* PlukM-*spn*, Δ*spn* PlukM-*gfp* were diluted, tested for MPO activity, and compared with Newman WT. Bars express SD with $n = 3$ (*A*–*D*). (*E*) Effect of *S. aureus* survival from isolated neutrophils after 60 min of phagocytosis. Statistical analysis determined by paired Student *t* test for *C* and *E*. Bars express SEM with $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$; ns, not significant.

Given the contribution of MPO in killing of phagocytosed *S. aureus*, we used the strong *lukM* promoter (22) and used the Newman strain to increase intraphagosomal SPIN production (WT PlukM-*spn*), thereby ensuring maximal levels of MPO inhibition in our bactericidal assays (Fig. 4*D*). We used the same plasmid to complement the isogenic *spn* KO (Δ*spn* PlukM-*spn*) and prepared a strain of the *spn* KO expressing GFP from the same plasmid (PlukM-*gfp*) to serve as a negative control. Supernatants from overnight cultures of these strains were compared with Newman WT for their ability to inhibit MPO (Fig. 4*D*). As predicted, the amount of SPIN produced in the WT PlukM-*spn* and Δ*spn* PlukM-*spn* was increased approximately fivefold relative to Newman WT, as judged by IC_{50} values. Importantly, these strains grew with similar kinetics in THB (Fig. S8*A*) and showed comparable phagocytosis by neutrophils at both 15- and 60-min time points (Fig. S8 *B* and *C*). Therefore, we used these three strains to detect differences in bacterial survival at 60 min following phagocytosis (Fig. 4*E*). Indeed, we observed a *spn*-dependent, significant difference in killing between the negative control strain Δ*spn* and the complemented strain that overexpressed *spn*. Although the difference between the Δ*spn* strain and the overexpressing WT strain was not statistically significant, it nevertheless showed a trend towards increased survival similar to the complemented strain. Taken together, these data show that SPIN is an important component to *S. aureus* survival of the oxidative killing mechanisms deployed within the neutrophil phagosome.

Discussion

Here we describe SPIN as a component of the *S. aureus* immune evasion arsenal that impairs bacterial killing by neutrophils. The phagocytosed bacteria must confront numerous antibacterial systems within the phagosome. Considering this as a potentially important site for execution of an immune evasion strategy, we set out to identify any previously unknown *S. aureus* proteins that inhibit components within neutrophil granules. By using an innovative phage-display approach that is limited to *S. aureus* secreted proteins (Fig. S1), we discovered that SPIN binds MPO with low nanomolar affinity and inhibits its activity (Fig. 1). Thus, together with the recently characterized neutrophil serine protease inhibitors, Eap, EapH1, and EapH2 (23), SPIN constitutes part of an elaborate staphylococcal innate immune evasion program that specifically targets the antibacterial enzymes stored within the azurophilic granules of neutrophils.

From experiments using promoter-GFP reporter bacteria, we observed that the promoter of *spn* is stimulated following uptake of *S. aureus* by neutrophils compared with bacteria outside neutrophils or in the first 2 h of growth in THB (Fig. 3 *B* and *C* and Fig. S5). Thus, SPIN is specifically up-regulated intracellularly, where MPO is located. A similar result was also observed when looking at mRNA levels (Fig. 3*A*), where the production of SPIN was increased over eightfold 30 min after phagocytosis. Moreover, there was no up-regulation observed when bacteria lacking the SaeR/S two-component regulatory system were used. The SaeR/S system plays an important role in neutrophil evasion (19) and regulates factors that reduce neutrophil ROS (13). SaeR/S is up-regulated comparably to *spn* (≈3- to 10-fold 30 min after phagocytosis) (20) and the promoter of SPIN contains the binding consensus sequence of SaeR (24). At least two of the 11 *ssl* genes (*ssl5* and *ssl8*) are regulated via the Sae system (25) and are located directly upstream of the SPIN gene. Thus, we propose that SPIN is largely regulated via the SaeR/S regulon and likely contributes to the SaeR/S-dependent reduction of neutrophil ROS (13).

By using a simplified system consisting of an enzymatic H_2O_2-generating system and Cl^- as a substrate for MPO, we found that SPIN had a clear dose-dependent and statistically significant effect on *S. aureus* survival (Fig. 4 *A* and *B*). However, in the complex and necessarily redundant process of bacterial killing within neutrophils, a multitude of oxygen radicals, proteases, and other enzymes must work in concert to ensure effective elimination of *S. aureus* cells. Although the contribution of MPO and its main product, HOCl, to *S. aureus* killing has been studied since the early 1970s (5), we and others believe this process remains incompletely understood (26). For example, it was previously believed that MPO is the main component for killing *S. aureus*, because neutrophils from MPO-deficient patients showed a log difference in bacterial survival after phagocytosis (5). Using the potent and cell-permeable MPO inhibitor, AZM198, we found that while untreated neutrophils kill 82 ± 5% of bacteria, MPO-depleted neutrophils are still able to kill 56 ± 16% (Fig. 4*C*). Thus, under the specific test conditions in our assays, roughly one-fourth of the staphylococcal killing is due to MPO. Nevertheless, when a strain of *S. aureus* overexpressing *spn* was compared with a *spn*-deleted counterpart (22) (Fig. 4*D*), we observed a statistically significant difference in bacterial survival dependent on SPIN (Fig. 4*E*). This not only confirms the relevance of SPIN-mediated inhibition of MPO in the physiological context, it also provides a starting point for reanalysis of previous studies where the presence of SPIN may have adversely affected data interpretation.

SPIN consists of a three α-helix bundle fold, which is commonly found among *S. aureus* innate immune evasion proteins, including Efb, Ecb (also called Ehp), Sbi, and SCINs (27). The triple-helix motif is a thermodynamically stable structure which can accommodate high sequence diversity and adapt to binding a wide variety of targets (28). However, this simple structural motif is typically modified by an N-terminal extension that imparts specific function to the protein in question (27). Indeed, this appears to be the case for SPIN as well, as is suggested by the cocrystal structure of SPIN/MPO (Fig. 2). To our knowledge, SPIN is unique as an example of a pathogen-derived protein whose role is to specifically bind and inhibit MPO. SPIN is not the only known inhibitor of MPO, however, as it has previously been shown that the host serum protein ceruloplasmin (CP) binds MPO with a K_D of ∼30 nM (29) and also blocks MPO activity (30). Examination of the crystal structure of CP bound to MPO (31) indicates that the mechanism through which CP inhibits MPO is conceptually distinct from that of SPIN. Indeed, even though the MPO active site appears to remain partly solvent accessible in the CP/MPO complex, CP attenuates MPO activity by disturbing essential transitions between the redox states of the peroxidase catalytic heme (32). In contrast, our structure of SPIN/MPO shows that SPIN forms a molecular plug that is likely to completely occlude access of substrates to the MPO active site (Fig. 2). Separately, while CP and SPIN share partial overlap in their binding sites on MPO and recognize their target with similar affinities, they display no obvious homology to one another at the sequence or structural levels. This suggests that the MPO-inhibitory capacity of SPIN has arisen through independent evolutionary processes from that of CP.

In conclusion, we identified an evasion protein called SPIN which is able to bind and inhibit human MPO. By solving the cocrystal structure of SPIN/MPO, we demonstrate the molecular mechanism of inhibition, via insertion of an N-terminal hairpin into the active site of MPO to block substrate access. We further show the expression of SPIN is up-regulated inside the neutrophil, where MPO is located, and that its presence helps in evading MPO-mediated killing. Various proteins secreted by *S. aureus* are known to be human-specific, which leads to restrictions in investigating this pathogen in animal models (8, 16). This seems to account for SPIN as well since it inhibits only human MPO and not MPO of other animals we have tested (Fig. S6). By adding this evasion molecule to the arsenal of previously identified evasion molecules, we can better understand the elaborate mechanism by which *S. aureus* evades killing through a multifaceted strategy that acts within the phagosomal compartment.

Materials and Methods

Bacterial Strains and Constructs. The bacterial strains and constructs used in this study are described in detail in *SI Materials and Methods*.

Identification, Expression, and Purification of SPIN. SPIN was subsequently produced in *Escherichia coli* and purified according to procedures described in *SI Materials and Methods*. Primers used in this study are listed in Table S2.

Binding Studies. Detailed procedures about ELISAs, SPR, and the AlphaScreen assay are described in *SI Materials and Methods*.

MPO Activity Assay. SPIN activity was determined by measuring the MPO activity via the redox indicator *o*-Dianisidine. For detailed procedures, see *SI Materials and Methods*.

Crystallization, Structure Determination, Refinement, and Analysis. A description of crystal cell constants, diffraction data quality, and properties of the final model for the SPIN/MPO complex can be found in Table S1. See *SI Materials and Methods* for detailed information about the SPIN/MPO crystal structure.

Analysis of *spn* and *agrA* Expression Following Exposure to Human Neutrophils. Gene expression was assessed using TaqMan RNA-to-C_T 1-*Step* Kit (Life Technologies). Informed consent for blood draw was obtained from all subjects, in accordance with the Declaration of Helsinki. Approval from the medical ethics committee of the University Medical Center Utrecht was attained (METC-protocol 07–125/C approved on 1 March 2010). For detailed procedures, see *SI Materials and Methods*.

Time-Lapse Microscopy and Phagocytosis Assay. Detailed information about time-lapse microscopy of promoter reporter USA300 bacteria and phagocytosis is described in *SI Materials and Methods*.

Bactericidal Assays. Bactericidal assays with MPO and GO or neutrophils is described in detail in *SI Materials and Methods*.

Graphical and Statistical Analyses. MPO activity analyses and statistical analyses were performed with Graphpad Prism v6. Statistical significance was calculated using ANOVA and Student's *t* test.

ACKNOWLEDGMENTS. We thank Franke A. Quee, Lindert Benedictus, Ruben Shrestha, Ping Li, and Kyler B. Pallister for experiments and statistical analysis. This research was supported by NIH Grants AI111203 and GM121511 (to B.V.G.), R01AI1090046 and PAR98-072 (to J.M.V.), and RR020185 for a fellowship award (to F.E.G.); and ZonMw Grant 205200004 from the Netherlands Organization for Health Research and Development (to J.A.G.v.S.). X-ray diffraction data were collected at Southeast Regional Collaborative Access Team 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract W-31-109-Eng-38.

1. Lowy FD (1998) *Staphylococcus aureus* infections. *N Engl J Med* 339:520–532.
2. Rigby KM, DeLeo FR (2012) Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol* 34:237–259.
3. Faurschou M, Borregaard N (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5:1317–1327.
4. Babior BM, Lambeth JD, Nauseef W (2002) The neutrophil NADPH oxidase. *Arch Biochem Biophys* 397:342–344.
5. Klebanoff SJ, Hamon CB (1972) Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. *J Reticuloendothel Soc* 12:170–196.
6. Kettle AJ, Winterbourn CC (1997) Myeloperoxidase: A key regulator of neutrophil oxidant production. *Redox Rep* 3:3–15.
7. Thwaites GE, Gant V (2011) Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? *Nat Rev Microbiol* 9:215–222.
8. Spaan AN, Surewaard BGJ, Nijland R, van Strijp JA (2013) Neutrophils versus *Staphylococcus aureus*: A biological tug of war. *Annu Rev Microbiol* 67:629–650.
9. Liu GY, et al. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* 202:209–215.
10. Cosgrove K, et al. (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* 189:1025–1035.
11. Kusch H, Engelmann S (2014) Secrets of the secretome in *Staphylococcus aureus*. *Int J Med Microbiol* 304:133–141.
12. Fevre C, et al. (2014) *Staphylococcus aureus* proteins SSL6 and SEIX interact with neutrophil receptors as identified using secretome phage display. *Cell Microbiol* 16:1646–1665.
13. Guerra FE, et al. (2016) *Staphylococcus aureus* SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production. *J Leukoc Biol* 100:1005–1010.
14. Fevre C, Scheepmaker L, Haas PJ (2017) Identifying bacterial immune evasion proteins using phage display. *Methods Mol Biol* 1535:43–61.
15. McCarthy AJ, Lindsay JA (2013) *Staphylococcus aureus* innate immune evasion is lineage-specific: A bioinfomatics study. *Infect Genet Evol* 19:7–14.
16. de Haas CJC, et al. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* 199:687–695.
17. Verkaik NJ, et al. (2009) Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of *Staphylococcus aureus*. *J Infect Dis* 199:625–632.
18. Palazzolo-Ballance AM, et al. (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J Immunol* 180:500–509.
19. Voyich JM, et al. (2009) The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis* 199:1698–1706.
20. Voyich JM, et al. (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *J Immunol* 175:3907–3919.
21. Björnsdottir H, et al. (2015) Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free Radic Biol Med* 89:1024–1035.
22. Vrieling M, et al. (2015) Bovine *Staphylococcus aureus* secretes the leukocidin LukMF' to kill migrating neutrophils through CCR1. *MBio* 6:e00335.
23. Stapels DA, et al. (2014) *Staphylococcus aureus* secretes a unique class of neutrophil serine protease inhibitors. *Proc Natl Acad Sci USA* 111:13187–13192.
24. Nygaard TK, et al. (2010) SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* 201:241–254.
25. Pantrangi M, Singh VK, Wolz C, Shukla SK (2010) Staphylococcal superantigen-like genes, ssl5 and ssl8, are positively regulated by Sae and negatively by Agr in the Newman strain. *FEMS Microbiol Lett* 308:175–184.
26. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM (2013) Myeloperoxidase: A front-line defender against phagocytosed microorganisms. *J Leukoc Biol* 93:185–198.
27. Serruto D, Rappuoli R, Scarselli M, Gros P, van Strijp JA (2010) Molecular mechanisms of complement evasion: Learning from staphylococci and meningococci. *Nat Rev Microbiol* 8:393–399.
28. Koymans KJ, Vrieling M, Gorham RD, Jr, van Strijp JAG (2016) Staphylococcal immune evasion proteins: Structure, function, and host adaptation. *Curr Top Microbiol Immunol* 6:23–27.
29. Bakhautdin B, Goksoy Bakhautdin E, Fox PL (2014) Ceruloplasmin has two nearly identical sites that bind myeloperoxidase. *Biochem Biophys Res Commun* 453:722–727.
30. Segelmark M, Persson B, Hellmark T, Wieslander J (1997) Binding and inhibition of myeloperoxidase (MPO): A major function of ceruloplasmin? *Clin Exp Immunol* 108:167–174.
31. Samygina VR, et al. (2013) Ceruloplasmin: Macromolecular assemblies with iron-containing acute phase proteins. *PLoS One* 8:e67145.
32. Chapman ALP, et al. (2013) Ceruloplasmin is an endogenous inhibitor of myeloperoxidase. *J Biol Chem* 288:6465–6477.
33. Fiedler TJ, Davey CA, Fenna RE (2000) X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution. *J Biol Chem* 275:11964–11971.
34. van den Berg S, et al. (2015) A human monoclonal antibody targeting the conserved staphylococcal antigen IsaA protects mice against *Staphylococcus aureus* bacteremia. *Int J Med Microbiol* 305:55–64.
35. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview version 2—A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191.
36. Carver TJ, et al. (2005) ACT: The Artemis comparison tool. *Bioinformatics* 21:3422–3423.
37. Bose JL, Fey PD, Bayles KW (2013) Genetic tools to enhance the study of gene function and regulation in *Staphylococcus aureus*. *Appl Environ Microbiol* 79:2218–2224.
38. Pang YY, et al. (2010) agr-Dependent interactions of *Staphylococcus aureus* USA300 with human polymorphonuclear neutrophils. *J Innate Immun* 2:546–559.
39. van Kessel KPM, van Strijp JAG, Verhoef J (1991) Inactivation of recombinant human tumor necrosis factor-alpha by proteolytic enzymes released from stimulated human neutrophils. *J Immunol* 147:3862–3868.
40. Rooijakkers SHM, van Wamel WJB, Ruyken M, van Kessel KPM, van Strijp JA (2005) Anti-opsonic properties of staphylokinase. *Microbes Infect* 7:476–484.
41. Bosse R, Illy C, Chelsky D (2002) *Application Note. Principles of AlphaScreen* (PerkinElmer Life Sciences, Boston).
42. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
43. Zwart PH, et al. (2008) Automated structure solution with the PHENIX suite. *Structural Proteomics High-Throughput Methods*, eds Kobe B, Guss M, Huber T (Humana Press, Totowa, NJ), pp 419–435.
44. Afonine PV, et al. (2010) Joint X-ray and neutron refinement with phenix.refine. *Acta Crystallogr D Biol Crystallogr* 66:1153–1163.
45. Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486–501.
46. Krissinel E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372:774–797.
47. Voyich JM, Sturdevant DE, DeLeo FR (2008) Analysis of *Staphylococcus aureus* gene expression during PMN phagocytosis. *Methods Mol Biol* 431:109–122.
48. Denys GA, Grover P, O'Hanley P, Stephens JT, Jr (2011) In vitro antibacterial activity of E-101 Solution, a novel myeloperoxidase-mediated antimicrobial, against Gram-positive and Gram-negative pathogens. *J Antimicrob Chemother* 66:335–342.
49. Bestebroer J, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. *Blood* 109:2936–2943.