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Staphylococcus aureus Formyl Peptide Receptor–like 1 Inhibitor (FLIPr) and Its Homologue FLIPr-like Are Potent FcγR Antagonists That Inhibit IgG-Mediated Effector Functions

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To evade opsonophagocytosis, Staphylococcus aureus secretes various immunomodulatory molecules that interfere with effective opsonization by complement and/or IgG. Immune-evasion molecules targeting the phagocyte receptors for these opsonins have not been described. In this study, we demonstrate that S. aureus escapes from FcγR-mediated immunity by secreting a potent FcγR antagonist, FLIPr, or its homolog FLIPr-like. Both proteins were previously reported to function as formyl peptide receptor inhibitors. Binding of FLIPr was mainly restricted to FcγRII receptors, whereas FLIPr-like bound to different FcγR subclasses, and both competitively blocked IgG-ligand binding. They fully inhibited FcγR-mediated effector functions, including opsonophagocytosis and subsequent intracellular killing of S. aureus by neutrophils and Ab-dependent cellular cytotoxicity of tumor cells by both neutrophils and NK cells. In vivo, treatment of mice with FLIPr-like prevented the development of an immune complex-mediated FcγR-dependent Arthus reaction. This study reveals a novel immune-escape function for S. aureus–secreted proteins that may lead to the development of new therapeutic agents in FcγR-mediated diseases. The Journal of Immunology, 2013, 191: 353–362.

The commensal bacterium Staphylococcus aureus is a leading cause of a wide variety of infections in humans and animals worldwide, ranging from mild superficial skin infections to fatal deep-seated infections that entail spread through the bloodstream and body (1). Its great success with regard to infection is explained by the numerous strategies that this commensal has developed to breach host immune responses (2, 3).

The principal line of defense against extracellular bacterial infection relies on phagocytes, including residential macrophages and immigrating neutrophils. These leukocytes recognize, remove, and destroy invading bacteria through phagocytosis. Bacterial recognition can occur directly via membrane-bound pattern recognition receptors, although many pathogens, including S. aureus, resist direct engulfment by phagocytes and require opsonization by complement (C3b, C3bi) or IgGs (IgA, IgG) for efficient phagocytosis. The phagocyte receptors for these opsonins, complement receptors CR1 and CR3, FcγRs, and leukocyte FcγRs, respectively, become activated upon ligand binding. They induce the initial uptake of the pathogen into a phagosome that subsequently fuses with lysosomes, resulting in the intracellular killing of the pathogen. An intracellular IgG receptor, the neonatal FcR (FcRn) (4), contributes to this process as well; however, mechanistic details remain to be elucidated.

To evade phagocytosis, S. aureus expresses a variety of both secreted and cell surface–associated immunomodulatory proteins. These proteins bind to key elements in this process and inhibit their proper function. Several of these abrogate effective bacterial opsonization by complement and/or IgG (5). SCIN (6), Efb, and its C-terminal homolog Ecb (7) are secreted proteins that target complement deposition on the bacterial surface by direct and indirect inhibition of C3 convertases. Sbi is an excreted and cell wall–anchored protein with a dual function, disturbing both complement and IgG opsonization (8, 9). Sbi binds to fragment C3d of complement factor C3 and to the constant domain (Fc) of IgG, thereby blocking interactions with FcγR and complement factor C1q. A similar inhibition of IgG Fc tail function, although targeting IgG1
exclusively, is provided by the secreted SSL10 (10). Staphylococcal protein A (SpA) is a cell wall–anchored IgG-binding (11) and anti-phagocytic protein expressed by most strains of \( S. \) aureus. Its inhibitory effect on phagocytosis is explained by the concept that SpA captures IgG onto the bacterial surface in a maloriented position, thereby preventing recognition by neutrophil FcγR. In addition to these blocking proteins, \( S. \) aureus also expresses an enzymatic inhibitor of bacterial opsonization, staphylokinase, which degrades the bacterium-bound opsonins, C3b and IgG, by activation of plasminogen into plasmin, an active serine protease (12). Staphylococcal immunomodulatory molecules that directly target the phagocyte opsonin receptors have not been described. In this study, we focus on FcγRs, the receptors for IgG.

Through binding of the Fc domain of IgG, FcγRs activate immune effector cells on which they are broadly expressed (13–15). FcγRs are members of the Ig superfamily. They possess an extracellular ligand–recognizing \( \alpha \)-chain consisting of two or three Ig-like domains, a transmembrane region, and an intracellular tail. Cross-linking of FcγRs by IgG-opsonized particles or immune complexes induces several cell type–dependent immune responses, ranging from phagocytosis, respiratory burst, and Ab-dependent cellular cytotoxicity (ADCC) to secretion of inflammatory mediators, enhancement of Ag presentation, and regulation of Ab production. Based on structural and biochemical differences they are divided in three distinct, but closely related, classes of FcγRs—FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16)—containing 12 isoforms that differ in cellular distribution and affinity for IgG. Monocytes and macrophages express members of all three FcγR classes: FcγRIa, FcγRIIa/b/c, and FcγRIIIa (on monocyte subset). The most abundant phagocyte, the neutrophil, constitutively expresses only two members of the FcγR classes: FcγRIIa and FcγRIIib. FcγRIIa is the predominant receptor involved in phagocytosis and binds IgG only in complexed or polymeric form. The FcγRIIa gene demonstrates allelic variation (16), resulting in two allotypes with differential affinity for IgG: FcγRIIa-H131(E27) and FcγRIIa-R131(Y27). Human neutrophils also express limited amounts of the inhibitory FcγRIIB (17, 18). FcγR class III contains two isoforms: the medium-affinity receptor FcγRIIIa, which is expressed on NK cells, macrophages, and some monocytes, and the GPI-anchored FcγRIIib, with a low affinity for IgG, which is constitutively expressed on neutrophils. For FcγRIIa, two functionally different allelic variants are known that differ at position 158 (V158 and F158) (16). FcγRIIib bears a polymorphism (16) at four positions in the extracellular region, resulting in two allotypes, neutrophil Ag (NA) 1 (R18 N47 D64 V88) and NA2 (S18 S47 N64 I88), which have different glycosylation patterns and interaction with IgG. The high-affinity IgG FcγRIa, which is expressed on monocytes, macrophages, and activated neutrophils, is structurally unique in that it contains three extracellular Ig-like domains, in contrast to the two found in FcγRIIIa and FcγRIIib.

In this study, we investigated the hypothesis that \( S. \) aureus evades opsonophagocytosis via the secretion of an FcγRIla inhibitory molecule. We describe the identification of two immunomodulatory homolog proteins secreted by \( S. \) aureus that target FcγR directly by recognizing epitopes in or near their IgG-binding domain. Both proteins were reported to be antagonists for the formyl peptide receptor (FPR) family. In this study, we demonstrate that these proteins are potent antagonists of FcγR-mediated effector functions in vitro, including opsonophagocytosis and ADCC, and in vivo.

**Materials and Methods**

**Abs and reagents**

The following anti-FcγR mAbs were used: PE-conjugated mAb 7.3 (FcγRII; Research Diagnostics), FITC-conjugated mAb FLI8.26 (FcγRII; BD), FITC-conjugated mAb IV.3 (FcγRII; American Type Culture Collection), FITC-conjugated mAb 30G8 (FcγRIII; BD), PE-conjugated mAb B73.1 (FcγRIII; BD), FITC-conjugated mAb 10.1 (FcγRI; BD), and PE-conjugated mAb MIP8a (FcγR). HRP-conjugated anti-His mAb was from Invitrogen, anti-polyHistidine mAb was from R&D Systems, and human IgG1k (HuMab-KLH) was from Gennab. Soluble fFcγRs of different subclasses—\( \alpha \)-I, \( \beta \)-I, \( \gamma \)-I, (H131 and R131), \( \beta \)-II, (V158 and F158), and \( \gamma \)-II (Na1 and Na2)—containing a polyhistidine tag were kindly provided by Gennab (19). Additional soluble FcγRIla, FcγR, and FcεR were from R&D systems. Human plasminogen (GenBank BA000018) was passed over a 25-ml “Reactive Red 120” ligand dye cross-linked 4% beaded agarose column (Sigma–Aldrich) attached to an Akta-FPLC system (GE Healthcare Life Sciences). After washing with PBS, the column was eluted into 2.5-ml fractions using 1 M NaCl. PMSF (1 mM) was added, and fractions were dialyzed in PBS for 18 h. Fractions were screened for activity by anti-FcγRIIab mAb staining on fresh human neutrophils. Active fractions were pooled, concentrated with a 10-kDa Centriprep (Amicon; Millipore), and separated on a GE Healthcare Superdex-75 gel filtration column into 2.5-ml fractions that were again screened for activity. Active fractions were pooled and concentrated using a 10-kDa Centriprep and stored at –20°C in small aliquots. Different fractions were precipitated with 20% TCA for 30 min on ice and analyzed on 15% SDS-PAGE (Mini-Protein II; Bio-Rad) by silver staining.

For affinity isolation, magnetic Cobalt-chelating beads (TALON Dynabeads; Invitrogen) were coated with recombinant His-tagged human CD32a (the extracellular domain Ala 36–Ile 218 of human FcγRIa; #1330-CD; R&D Systems). Fifty microliters of beads was washed twice with PBS containing 0.1% Triton X-100 (PBS-Triton) and incubated for 30 min with 100 μl 200 μg/ml His-tagged CD32a in PBS. Beads were washed three times with PBS-Triton and incubated with purified supernatant for 18 h at 4°C under gentle rotation in a total volume of 400 μl. Supernatant was discarded, and beads were washed three times with PBS-Triton, suspended in 30 μl SDS-PAGE sample buffer for 15 min, and heated for 2 min at 100°C. The sample was centrifuged briefly (10 s at 10,000 \( \times \) g), and the supernatant was analyzed on 15% SDS-PAGE by silver staining. Bands were excised and sent for protein identification at the Department of Bio-molecular Mass Spectrometry, Utrecht Institute for Pharmaceutical Sciences, Utrecht, The Netherlands.

The recombinant staphylococcal proteins FLIPr, FLIPr-\( \alpha \)-like, and CHIPS were expressed and purified from Escherichia coli, as described (20–22).

**Surface-enhanced laser desorption ionization time-of-flight mass spectrometry**

For identification by mass, a Ciphergen (Bio-Rad) IMAC30 ProteinChip Array was used that incorporates nitrocellulose acid groups forming stable complexes with metal ions. After loading the array with nickel sulfate, 10 μg/ml His-tagged soluble FcγRIa was added and subsequently incubated with semipurified concentrated staphylococcal supernatant. The array was washed with PBS, rinsed with water, air dried, and treated with a saturated solution of sinapinic acid, an energy-absorbing molecule that assists in desorption and ionization. After air drying, the array was analyzed using the Ciphergen ProteinChip System Series 4000 read at a setting optimized for single molecule mass change. Spectra were externally calibrated, baseline corrected, and normalized to total ion current within a mass/charge (m/z) range of 1,500–50,000 Da.

**Cells and flow cytometry staining**

The mouse P388D1 macrophage cell line was maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS. Human neutrophils and mononuclear cells were isolated from heparinized blood as described (21). Cells were incubated with staphylococcal supernatants (30% v/v) or purified recombinant proteins for 0–10 min on ice, washed, and subsequently stained with fluorescent antibodies. Fluorescent intensities were measured by flow cytometry and expressed relatively to control cells not exposed to supernatant or recombinant protein. FLIPr and FLIPr-\( \alpha \)-like were labeled with FITC, as described (21, 22), and incubated with leukocytes in the presence of specific markers for monocytes (PE-labeled anti-CD14). T cells were incubated with staphylococcal supernatants (30% v/v) or purified recombinant proteins for 0–10 min on ice, washed, and subsequently stained with fluorescent monoclonal antibodies. The presence of specific markers for neutrophils (PE-labeled anti-CD14), T cells (allophycocyanin-labeled anti-CD3), B cells (PE-labeled anti-CD19), or NK cells (PE-labeled CD56 and allophycocyanin-labeled anti-CD56). Cells were gated by their scatter and marker profile.
Evaluation of FcγR binding and Ig inhibition by ELISA

To detect binding of staphylococcal proteins to FcγR subclasses, MaxiSorp plates (Nunc) were coated overnight at 4°C with 100 μl FLIPr, FLIPr-like, or chemotaxis inhibitory protein of Staphylococcus aureus (CHIPS) at 1 μg/ml in PBS. Plates were washed three times with PBS supplemented with 0.05% v/v Tween 20 and incubated with a serial dilution of different soluble FcγR proteins containing a polyhistidine tag in PBS supplemented with 0.05% v/v Tween 20 and 0.2% v/v BSA. After 2 h of shaking (300 rpm) at room temperature, plates were washed and incubated for 90 min with HRP-conjugated anti-His mAb to detect FcγR binding. Inhibition of Ig binding to the different FcγR subclasses by staphylococcal proteins was measured with another ELISA. Plates were coated overnight with 4 μg/ml anti-polystartide mAb, washed, and incubated with 2 μg/ml solubilized His-tagged FcγR for 90 min. The plates were washed and incubated with a serial dilution of the staphylococcal proteins. After washing, the different subclasses of FcγR were probed with an optimal concentration of human monoclonal IgG1 (HuMab-KLH) that was detected with HRP-conjugated anti–human Fc. Statistical analysis was performed using a two-tailed Student t test.

Phagocytosis and killing

Phagocytosis was measured with FITC-labeled staphylococci (6). In a 96-well plate, bacteria were mixed with a concentration range of human serum (pooled from 15 donors), complement-inactivated serum (30 min at 56°C), or purified IgG (by Protein G affinity chromatography from the same serum pool) for 15 min at 37°C on an orbital shaker. Subsequently, neutrophils or macrophages, with or without inhibitor, were added at a 10:1 bacterium/cell ratio and incubated for 15 min at 37°C on a shaker (750 rpm). The reaction was stopped with ice-cold paraformaldehyde (1%), and cell-associated fluorescent bacteria were analyzed by flow cytometry. Phagocytosis is defined as the percentage of cells with a positive fluorescent signal. To compare the experiments, the values are expressed relative to the second highest opsonin concentration of control cells without inhibitor protein. Killing of staphylococci was determined by colony plate counting. Bacteria were opsonized for 15 min with 1% complement-inactivated serum, mixed with neutrophils at a 1:10 ratio, and incubated for 1 h at 37°C while shaking. A 50-μl sample was diluted into 2.5 ml ice-cold water (pH 11), vortexed, and incubated for 5 min on ice to lyse the neutrophils. The remaining viable bacteria in each reaction mixture were counted by plating 50 μl onto nutrient agar plates, followed by overnight incubation at 37°C. Controls consisted of nonopsonized bacteria and bacteria without neutrophils.

IgG transcytosis

Twelve-millimeter polycarbonate Transwell filters (0.4 μm pore size; Costar/Corning) were incubated with 5 × 10^5 human choriocarcinoma cells (JAR), grown overnight to confluence, and washed with PBS; medium was replaced with 1.5 ml fresh medium basolaterally and 0.5 ml apically (IMDM [pH 7.4], supplemented with l-glutamine, 10% FCS, and penicillin/streptomycin). Mixtures of IgG contained 125 pg/ml streptavidin-HRP (SA-HRP) to assess background transport. Apical to basolateral transport was calculated using the following formula: \( \text{IgG_{basolateral}} \times 1.5 \text{ ml} \) / (IgG_{apical} × 0.5 ml) × 100%.

ADCC

ADCC assays against 51Cr-labeled A1207 target cells were performed as described (23, 24). In short, isolated neutrophils or NK cells (from PBMCs using the EasySep Human NK cell Enrichment Kit; STEMCELL Technologies) were preincubated with 3 μg/ml inhibitory protein. Sensitizing Abs (1 μg/ml) were added to microtiter plates, and ADCC was induced by adding effector and target cells at an E:T ratio of 80:1 for neutrophils and 10:1 for NK cells. The plates with NK cells as effector cells were centrifuged for 5 min at 300 rpm. After 4 h at 37°C, [51Cr] release from triplicates was measured (cpm). The percentage of cellular cytotoxicity was calculated using the following formula: percentage of specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100%. Maximal [51Cr] release was determined in the presence of 5% Triton X-100, and basal lysis was determined in the absence of sensitizing Abs and effector cells. Data are expressed as relative lysis compared with buffer-treated cells toward mAb-coated targets. Statistical analysis was performed using a two-tailed Student t test.

Peritoneal Arthus reaction

BALB/c mice were injected i.v. with 100 μl OVA (20 mg/kg of body weight; Sigma-Aldrich), immediately followed by i.p. injection of 200 μl rabbit anti-OVA IgG (800 μg/mouse; MP Biomedicals) in sterile PBS, as described (25). For inhibition experiments, 100 μl FLIPr-like was administered i.v. and i.p. (600 μg/ml) 30 min before initiation of the Arthus reaction. Mice in different treatment groups were killed 6 h after onset of the reaction. The peritoneal cavity was lavaged with 6 ml ice-cold PBS/0.1% BSA. Peritoneal cells were washed once with PBS, and neutrophil numbers were calculated from cytopsin slides stained with Diff-Quick. Neutrophil numbers per square millimeter were calculated from 20 microscopic fields. Animal care was provided in accordance with National Institutes of Health guidelines. Animal studies were approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee.

Statistical analysis

Significant differences (p < 0.05) were identified with Student t tests and one-way ANOVA using GraphPad Prism 5.0 software (GraphPad). Data are expressed as mean ± SD or SEM.

Results

Identification of a putative staphylococcal FcγRIIa inhibitor

To investigate potentially secreted staphylococcal FcγRIIa antagonists, culture supernatants of various clinical and laboratory S. aureus strains were screened for their ability to competitively inhibit specific neutrophil FcγRIIa staining by flow cytometry. The anti-FcγRII mAb clone 7.3 was used because of its specificity for an epitope in the IgG binding site of the receptor (26). Several staphylococcal supernatants inhibited mAb 7.3 binding to FcγRIIa on neutrophils (data not shown). Supersaturated of S. aureus strain N315 was among the most potent (Fig. 1A) and was selected for purification of the inhibitory compound. Lindig dye chromatography, gel permeation, and anion exchange chromatography were performed, and activity of the fractions was monitored by mAb 7.3 neutrophil staining. Using a chip coated with soluble rFcγRIIa, combined with mass detection in a surface-enhanced laser desorption/ionization time-of-flight system, identified a 12.3-kDa mass peak in the semipurified staphylococcal supernatant (Fig. 1B). Affinity isolation on magnetic beads, coupled with soluble FcγRIIa, was used to obtain a final active preparation containing one single nearly homogeneous protein band ~13 kDa on SDS-PAGE (Fig. 1C). Gel extraction and subsequent mass spectrometry identified the protein as FLIPr. FLIPr, a secreted staphylococcal protein of 105 aa with a mass of 12.3 kDa, is a potent inhibitor of the chemotaxis receptor FPR2 (formerly annotated as formyl peptide receptor-like 1 or FPR1L) (21). Initially, FLIPr was discovered based on its 49% sequence homology with CHIPS (chp), the chemotaxis inhibitory protein of S. aureus (20).

To confirm that FLIPr was the active FcγRIIa inhibitory compound present in the staphylococcal supernatant, recombinant FLIPr was tested for its effect on neutrophil FcγRIIa staining. Like the supernatant, recombinant FLIPr inhibited binding of mAb 7.3 to neutrophils, with an IC50 of 48.4 ng/ml (range, 33.5–69.8 ng/ml) (Fig. 1D). The recombinant homolog of FLIPr, FLIPr-like (104 aa with ~72% identity) (22), was ~10-fold more active in FcγRIIa blocking compared with FLIPr, with an IC50 of 4.9 ng/ml (range, 3.7–6.5 ng/ml). The related staphylococcal protein CHIPS did not affect FcγRIIa staining. Because of its structural and functional similarities, CHIPS was used as control protein in further experiments.

FLIPr and FLIPr-like bind different FcγR isoforms and block IgG binding

A capture ELISA was performed to confirm that FLIPr and FLIPr-like directly interact with FcγRIIa, as well as to analyze the specificity for other Fc(γ)Rs. Different recombinant soluble FcγRs and their polymorphic isoforms containing a polyhistidine tag (Ia, Ila–H131 and Ila–R131, Iib, Ila–V158 and Ila–F158, and Iib–NA1 and Iib–NA2) were evaluated for their relative binding to the coated...
staphylococcal proteins FLIPr, FLIPr-like, and CHIPS. Both FLIPr and FLIPr-like bound to soluble FcγRIIa in ELISA (Fig. 2B, 2C). In contrast to FLIPr-like, FLIPr demonstrated differential binding to the two allelic variants of FcγRIIa that differ at position 131, which is located in the ligand-binding region and is crucial for IgG binding (15). FLIPr bound preferentially to the high-affinity isoform FcγRIIa-H131, whereas binding to the low-affinity R131 isoform was almost negligible. The highly homologous inhibitory receptor FcγRIIb, whose ectodomain differs at 10 positions from FcγRIIa (11 for the FcγRIIa-H131 variant) (27), including two (or three for FcγRIIa-H131) amino acids involved in IgG binding, was bound equally well by FLIPr and FLIPr-like (Fig. 2D). With regard to the other FcγR subclasses, FLIPr did not bind significantly to FcγR class I and III receptors (Fig. 2A, 2E–H) and could be considered class II specific. In contrast, FLIPr-like bound to FcγRIla (Fig. 2D) and differentially to FcγRIIIa-V158 and FcγRIIIa-F158, an amino acid also involved in IgG binding (Fig. 2E, 2F). The two isoforms of FcγRIIib, differing by six ectodomain amino acids from FcγRIIIa-F158 (and 5 aa from FcγRIIIa-V158), of which two are located in IgG-binding regions (23, 24, 28), were both bound by FLIPr-like, but only minimally in the case of FcγRIIIb-NA1. CHIPS did not bind to any of the soluble FcγRs. Furthermore, none of the staphylococcal proteins bound to soluble FcεR, the receptors for IgA and IgE, respectively, in ELISA (data not shown). Because all FcγRs are composed of Ig-like domains strongly resembling the domains of IgG, we used ELISA to test whether FLIPr and FLIPr-like bound to IgG molecules, as well. In contrast to Mac-1, a streptococcal FcγR-binding protein that does bind IgG (29), neither FLIPr nor FLIPr-like bound IgG in ELISA (data not shown).

To investigate whether FLIPr and FLIPr-like interfere with FcγR–IgG interactions, an inhibition ELISA was performed. FcγRs were incubated with staphylococcal proteins, and relative

**FIGURE 1.** Identification of FLIPr as the secreted staphylococcal protein that inhibits FcγRIIa staining. (A) Inhibition of human neutrophil staining of FcγRIIa by serial dilutions of the supernatant from *S. aureus* strain N315. (B and C) Analysis of the protein(s) purified from *S. aureus* N315 culture supernatant using immobilized soluble FcγRIIa. (B) Surface-enhanced laser desorption/ionization time-of-flight profile of enriched active supernatant fraction on noncoated IMAC chip (upper panel) and IMAC chip coated with soluble FcγRIIa (lower panel). Mass of soluble FcγRIIa and the major specific peak are indicated. (C) Silver nitrate staining of the same active fraction captured with FcγRIIa-coated magnetic Tonal beads resolved by SDS-PAGE. (D) Inhibition of neutrophil FcγRIIa staining by serial dilutions of the purified recombinant staphylococcal proteins FLIPr, FLIPr-like, and CHIPS. FcγRIIa staining of antagonist-treated cells by mAb 7.3 (A, D) is expressed relative to the fluorescence value of buffer-treated cells (mean ± SEM of three experiments).
binding of recombinant human IgG1 to FcγR was determined. FLIPr and FLIPr-like efficiently prevented binding of human IgG1 to the FcγRs (Fig. 3), in accordance with their specific binding. The control protein CHIPS did not inhibit FcγR–IgG binding. In summary, FLIPr seems to be class II restricted and preferentially inhibiting IgG binding to FcγRIIa-H131 (Fig. 3B), the predominant receptor in phagocytosis. In contrast, FLIPr-like displays a broader profile and inhibits FcγR–IgG interactions for all FcγRs, with the exception of FcγRIIIb (Fig. 3G).

**FLIPr and FLIPr-like bind to human FcγR-expressing leukocytes and recognize epitopes involved in ligand binding**

In earlier studies, we demonstrated concentration-dependent binding of FLIPrFITC and FLIPr-likeFITC to neutrophils and CD14+ monocytes (21, 22) (Supplemental Fig. 1A, 1B), cells known to express both FPRs and FcγRs. The binding of FLIPr-likeFITC could be inhibited by high-affinity FPR1 ligand, peptide WKYMVM, but only partially, suggesting unrecognized residual FcγR staining. Furthermore, FLIPrFITC and FLIPr-likeFITC bound to FPR1 but FcγR-expressing CD19+ B cells and CD3+CD56+ NK cells (Supplemental Fig. 1D, 1E). Neither protein bound to FPR2 and FcγR-CD3+ T cells (Supplemental Fig. 1C).

To further investigate the binding site of FLIPr and FLIPr-like on the FcγR, additional mAb-competition studies were performed. In addition to anti-FcγRII mAb 7.3, FLIPr and FLIPr-like inhibited neutrophil binding of the more FcγRIIa-specific mAb IV.3, which also recognizes D2 epitopes involved in IgG interactions (26) (Fig. 4A, 4B). In contrast, neutrophil staining with the IgG-blocking anti-FcγRII mAb FLI8.26, which binds critical, although distinct, epitopes in D2 (26), was not affected by FLIPr and FLIPr-like (Fig. 4C). Similar patterns of competitive inhibition of FcγRII mAb staining by FLIPr and FLIPr-like were observed on human

**FIGURE 2.** FLIPr and FLIPr-like bind to different FcγR isoforms. Comparison of His-tagged soluble FcγRIa (A), FcγRII isoforms (IIa-H131, IIa-R131 and IIb) (B–D), and FcγRIII isoforms (IIIa-V158, IIIa-F158, IIb-NA1, and IIb-NA2) (E–H) for binding to FLIPr, FLIPr-like, and CHIPS by ELISA. Data are mean ± SEM of three independent experiments.

**FIGURE 3.** FLIPr and FLIPr-like compete for IgG1 ligand binding. Comparison of His-tagged soluble FcγRIa (A), FcγRII isoforms (IIa-H131, IIa-R131, and IIb) (B–D), and FcγRIII isoforms (IIIa-V158, IIIa-F158, IIb-NA1, and IIb-NA2) (E–H) for ligand inhibition by ELISA. Inhibition of human IgG1 binding to plates coated with FcγR in the presence of serial dilutions of FLIPr, FLIPr-like, and CHIPS is expressed as the percentage of buffer IgG1 binding. Data are mean ± SEM of three independent experiments.
monocytes and CD19+ B cells (Supplemental Fig. 2A–F). However, on B cells (expressing only FcγRIIb), binding of anti-FcγRII mAb FLI8.26 was inhibited by FLIPr (Supplemental Fig. 2C). Monocyte staining with the partially blocking anti-FcγRI mAb 10.1 (30, 31) was not affected by FLIPr or FLIPr-like (Supplemental Fig. 2G). Because FLIPr-like was shown to bind and block soluble FcγRIIa in ELISA, competitive inhibition of anti-FcγRII mAb 3G8-mediated staining of human NK cells expressing FcγRIIIa (and FcγRIIc for some individuals) was analyzed. mAb clone 3G8, which does not distinguish between FcγRIIa and FcγRIIib, binds to the putative FG loop within D2 (32), the major binding site for IgG. FLIPr-like efficiently prevented the mAb 3G8-mediated FcγRIIa staining on NK cells (Fig. 4D). In contrast to the ELISA results, FLIPr inhibited this mAb 3G8-mediated NK cell staining even more strongly. NK cell staining with the FcγRII-specific mAb clone B73.1, which recognizes epitopes distinct from the IgG binding site restricted to the membrane distal domain 1 (D1) (32), was not inhibited by the staphylococcal proteins (Fig. 4E). Both FLIPr and FLIPr-like demonstrated inhibition of anti-FcγRII mAb 3G8-mediated staining of neutrophils (expressing FcγRIIIB and FcγRIIa) (Fig. 4F); this was in partial contrast to the ELISA results that indicated no binding of FLIPr to FcγRIIib. Binding of an IgA-blocking anti-FcεR mAb to monocytes and neutrophils was not affected by FLIPr or FLIPr-like (Supplemental Fig. 2H, 2I). The control protein CHIPS did not interfere with leukocyte anti-FcγR mAb binding in any of the experiments. Altogether, FLIPr and FLIPr-like inhibited anti-FcγR mAb from binding to epitopes involved in FcγR-IgG interactions, indicating that the staphylococcal proteins bind FcγRs in or near their IgG Fc-binding regions within D2.

**Evasion of FcγR-mediated phagocytosis and killing**

To test whether FLIPr and FLIPr-like could inhibit FcγR-mediated effector functions, we performed phagocytosis experiments. Fluorescently labeled staphylococci were opsonized with purified human IgG, complement-inactivated human pooled serum, or untreated human pooled serum with intact complement activity. Human neutrophils were incubated with the staphylococcal proteins at a concentration of 3 μg/ml, and phagocytosis was determined at increasing opsonin concentrations. Both FLIPr and FLIPr-like efficiently inhibited FcγR-mediated phagocytosis of staphylococci opsonized with purified IgG (Fig. 5A). Staphylococci opsonized with complement-inactivated serum were also fully protected from phagocytosis by FLIPr-like, whereas FLIPr did so less strongly (Fig 5B). In the presence of complement, the inhibitory effects of FLIPr and FLIPr-like on phagocytosis were only observed at the lower serum concentrations of 0.5 and 0.25% (Fig. 5C). The inhibitory proteins were both effective after a short preincubation with the neutrophils and when serum (or IgG), inhibitory protein, cells, and, finally, bacteria were immediately mixed together. Titration of staphylococcal protein concentrations demonstrated the 2-fold (2.3 ± 0.4) higher potency of FLIPr-like, which up to 0.75 μg/ml completely inhibited FcγR-mediated neutrophil phagocytosis in the presence of complement-inactivated serum (Supplemental Fig. 3). The reduced neutrophil bacterial uptake observed in the phagocytosis experiments was reflected by increased *S. aureus* survival in bacterial-killing experiments in the presence of FLIPr and FLIPr-like (Fig. 5D). FLIPr-like did not interfere with the function of FcRn, the structurally distinct, other IgG receptors (Fig. 6C). NK cell FcγRIIIa (and FcγRIIc in some individuals)-mediated ADCC via IgG1 was inhibited by FLIPr-

**Inhibition of FcγR-mediated tumor cell killing**

Next, we tested whether FLIPr and FLIPr-like also inhibited additional FcγR-dependent cellular responses, such as ADCC, using specific human mAbs against tumor targets. Neutrophil FcγRIIa-mediated ADCC through panitumumab (IgG2; Fig. 6A) and cetuximab (IgG1; Fig. 6B), both directed against the epidermal growth factor receptor, were almost completely prevented by 3 μg/ml FLIPr and FLIPr-like, reaching the level of lysis in the absence of sensitizing mAb (no Ab). In agreement with ELISA and competitive mAb-staining data, human IgA-mediated neutrophil FcγRI-dependent ADCC was not affected by the staphylococcal FcγR inhibitors (Fig. 6C). NK cell FcγRIIIa (and FcγRIIc in some individuals)-mediated ADCC via IgG1 was inhibited by FLIPr-

**FIGURE 4.** FLIPr and FLIPr-like bind to leukocyte FcγR via epitopes in or near the ligand-binding domain. Human neutrophils (A–C, F) and CD3+/-CD56+ NK cells (D, E) were incubated with 3 μg/ml staphylococcal protein and analyzed for cell surface anti-FcγR mAb staining by flow cytometry. Relative binding of FcγRII mAb 7.3 (A), IV.3 (B), and FLI8.26 (C) and FcγRIII mAb 3G8 (D, F) and B73.1 (E) compared to buffer-treated cells. Data are mean ± SEM of three independent experiments. *p < 0.05, versus buffer-stained cells, two-tailed Student t test.
like, but not by FLIPr (Fig. 6D), in accordance with their selective binding preferences to FcγRII and FcγRIII in ELISA (Fig. 2). CHIPS did not interfere with neutrophil- or NK cell–mediated ADCC. Taken together, FLIPr and FLIPr-like effectively inhibited phagocyte activation in vitro by preventing engagement and cross-linking of FcγR by IgG opsonized to target cells.

FLIPr-like prevents Arthus reaction in mice

To investigate the in vivo potency of the staphylococcal FcγR antagonists, the immune complex–mediated passive Arthus reaction model in mice was used (33). In this model, i.p. generation of immune complexes initiates a type III hypersensitivity reaction by FcγR-dependent influx of neutrophils. We confirmed that both proteins inhibited the murine FcγR-mediated phagocytosis using the mouse macrophage cell line P388D1. Similar to human neutrophils, mouse phagocytes were inhibited by FLIPr and FLIPr-like (Fig. 7A). Furthermore, FLIPr and FLIPr-like effectively inhibited phagocytic activation in vitro by preventing engagement and cross-linking of FcγR by IgG opsonized to target cells.

Discussion

In this article, we describe a previously unrecognized role for the two homologous staphylococcal immune evasion proteins FLIPr and FLIPr-like. They bind FcγR and abrogate their IgG-recognizing function. FcγR-bearing immune cells that are involved in crucial antibacterial defense mechanisms lose their capacity to specifically recognize pathogenic targets. S. aureus demonstrates a novel strategy to evade host immunity. Mac-2 is an FcγR-binding protein secreted by Streptococcus pyogenes. It bound soluble FcγRII and FcγRIII and competed with IgG binding (29), as well as prevented the generation of reactive oxygen species by opsonized latex beads. However, in a whole-blood survival assay of S. pyogenes, only Mac-2 with intact IgG endopeptidase activity facilitated bacterial survival (35). To our knowledge, no other bacterial FcγR antagonist has been described; therefore, this is the first study to demonstrate a bacterial FcγR-binding protein...
that inhibits FcyR-mediated effector functions in vitro, as well as in vivo.

FLIPr and FLIPr-like are secreted proteins encoded by genes that cluster with other immune-evasion molecules, including SCIN, Efb, and Ecb, in the immune evasion cluster II (7). The genomes of all sequenced S. aureus strains contain either the gene encoding FLIPr or FLIPr-like, suggesting that the function of these homologs is essential to S. aureus survival and replication (36). They both exhibit dual functionality, as is observed for other S. aureus immunomodulatory molecules, such as CHIPS, which interacts with the FPR and C5aR (20), and SSL7, which binds both C5 and IgA (37). We previously reported their first recognized function in immune evasion, which is directed at the escape from neutrophils and monocytes by blocking FPR-mediated cell activation and chemotaxis. In this report, we demonstrate that FLIPr and FLIPr-like also bind FcyR. Both receptor families are found on macrophages, peripheral blood neutrophils, and monocytes; however, in contrast to FcyRs, FRPs are not expressed on resting NK cells (38).

It is likely that different regions within FLIPr and FLIPr-like are responsible for the inhibition of the FPR versus FcyR family of receptors that share no sequence or structural homology. Cross-talk between FPR and FcyR is described. Activation of FPR by FMLF, the archetype formylated peptide originating from growing bacteria, results in an increased surface expression of many cellular receptors, including FcyR (39, 40). Furthermore, it was suggested that signaling of FcyRIIib might involve FPR and that activation of FcyRIIib on human neutrophils modulates FMLF binding and migration (41). It is not known whether FLIPr and FLIPr-like can bind to both FPR2 and FcyR on phagocytes simultaneously, thereby inducing colocalization.

Despite their high degree of homology, FLIPr was almost exclusively restricted to class II receptors, with a preference for the high-affinity polymorphic allotype of the receptor FcyRIIa-H131. FLIPr-like bound to most FcyR isoforms tested, including FcyRI, which is expressed on monocytes, macrophages, and transiently on neutrophils during infection (42). FcyRIIa is the predominant receptor in ADCC by NK cells, although it is involved in phagocytosis by mononuclear cells, as well. FcyRIIia is also solely bound by FLIPr-like.

All FcyRs have similar modes of binding to IgG via sites that are structurally conserved (15, 43). X-ray crystallography, together with mutagenesis studies and homology modeling, demonstrated that the FcyR surface, which interacts with IgG, is primarily located in the extracellular D2 of all FcyRs, including FcyRI, which contains three (instead of two) extracellular domains. The FcyR binding site on IgG is primarily formed by residues in the lower hinge region and three segments of the CH2 domain. The fact that FLIPr and FLIPr-like bind to the FcyR, as well as antagonize FcyR–IgG interactions, was already indicated by the fact that both proteins competitively inhibited neutrophil staining with mAb clone 7.3, which was characterized as recognizing epitopes within the IgG binding site of FcyRII (26). Subsequently, other mAbs, binding to distinct epitopes in or near the IgG binding site of both
FcγRIIa (mAb IV.3) and FcγRIII (mAb 3G8), also were inhibited. mAb clone B73.1, which binds epitopes on D1 outside of the IgG-ligand binding domain of FcγRIII, was not inhibited by FLIPr or FLIPr-like on NK cells. The lack of inhibition of ligand-blocking anti-FcγRII mAb FL18.26 can be explained by the fact that this mAb is a member of another cluster (II) of mAb that binds the receptor via distinct D2 epitopes. The inhibition of anti-FcγRII mAb 3G8 from binding to neutrophils and NK cells by FLIPr, without any binding to class III receptors by ELISA, is difficult to explain. It may be that this mAb (mouse IgG1) binds with its Fc tail to FcγRIIC, also expressed by NK cells (donor dependently) (44). This discrepancy correlates with binding of fluorescent FLIPr to NK cells that do not express FPR2, the other FLIPr-binding surface receptor (21). A second indication that both proteins bind to or near the IgG-recognition region of the FcγR was reflected in the fact that the binding of FLIPr and FLIPr-like was influenced by single polymorphic differences in the FcγR ectodomain IgG-binding amino acids (FcγRIIa-131 and FcγRIIIa-158). The ELISA data with regard to recombinant expressed soluble receptor variants show a good correlation between protein binding and functional inhibition of IgG1–ligand interactions. However, a disadvantage is the variation in affinity between the FcγRs for monomeric IgG (45), although the IgG1 concentration was optimized for each FcγR class.

The most important FcγR-mediated immune response in the battle against *S. aureus* infection is phagocytosis and subsequent killing. FcγRIIa-mediated phagocytosis of IgG-opsonized bacteria by human neutrophils was efficiently prevented by FLIPr and FLIPr-like. FLIPr-like inhibition was stronger than that of FLIPr, which could be due, in part, to the (unknown) variation in donor FcγRIIa genotype. FLIPr is a more efficient FcγRIIa-H131 inhibitor. FcγR polymorphisms have been associated with susceptibility to or severity of autoimmune and infectious disease (16), van Mirre et al. (46) showed that the ratio of activating FcγRIIa/ inhibitory FcγRIIB2 mRNA in neutrophils varies among individuals, is associated with FcγR2b promoter haplotypes, and is accompanied by differences in the responsiveness of these cells to IgG complexes. However, the same investigators showed that, in contrast to the much smaller IgG dimers and aggregates, no significant difference was observed for phagocytosis of IgG-opsonized FITC-labeled *S. aureus*. The inhibition of phagocytosis in serum-containing active supplement was only observed at low serum concentrations. This could indicate that, under conditions with sufficient serum (>1%), the phagocytic process operates mainly via the complement receptors, and only at low serum concentrations is the process dependent on FcγR. Therefore, one could argue that these proteins contribute minimally to the immune-evasion arsenal of staphylococci. However, the relative amounts of individual complement components and IgG levels in the interstitial tissue outside of the blood vessels are unknown and could be very different, consequently skewing toward FcγR dependency. In addition, *S. aureus* secretes several potent inhibitors of the complement system (5). In contrast to SpA, SSL10, and Shi, which bind to IgG and inhibit phagocytosis, FLIPr and FLIPr-like directly target the relevant receptor of the phagocytosis process.

The ability of FLIPr and FLIPr-like to block FcγR-mediated functions was also shown in the ADCC experiments that used well-defined monoclonal human subclass IgG molecules to initiate tumor cell killing. Both FLIPr and FLIPr-like completely blocked neutrophil-mediated ADCC without interfering with the IgA-dependent killing of the same tumor cell target. The NK cell FcγRIIIa-mediated ADCC was completely abolished by FLIPr-like, whereas FLIPr was ineffective. This correlates with the ELISA data demonstrating lack of binding of FLIPr to the soluble extracellular part of FcγRIIIa. In addition to its role in antitumor immunity, ADCC is an important immune response against virally infected cells. Although speculative, by inhibiting ADCC and consequently suppressing local antiviral immunity, FLIPr-like might play a pathogenic role in the observed coinfections of *S. aureus* and influenza virus (47).

In vitro, FLIPr-like proved to be an effective inhibitor of mouse FcγR-mediated phagocytosis. Mouse and human FcγR are structurally very similar and exhibit identical modes of interaction with IgG (15). FcγRs play a central role in the pathogenesis of immune complex–based inflammation and disease, such as Ab-induced arthritis, immune anemia, and immune complex glomerulonephritis (25, 48). The reverse passive Arthus reaction in mice is a well-established in vivo model of immune complex–mediated inflammatory disease that relies on C5a/C5aR and the activating FcγR (33, 49). FcγR-γ-chain−/− mice, as well as C5aR−/− mice, exhibited abolished neutrophil influx in the peritoneum (25). Intervention with C5 activation by the staphylococcal protein EcB or SSL7 also completely inhibited neutrophil accumulation in the peritoneum (7, 50). In this study, we demonstrate that administration of FLIPr-like blocked neutrophil influx in the peritoneum by inhibition of the other arm of this inflammatory model.

*Staphylococcus aureus* secretes a set of two homologous proteins that effectively bind and neutralize FcγR-mediated effector mechanisms. Because these proteins bind several FcγR isoforms expressed on different leukocytes, these proteins are a valuable tool in dissecting the general contribution of FcγR to inflammation and infection. Whether FLIPr and FLIPr-like contribute to the virulence of *S. aureus* needs to be addressed in an appropriate in vivo infection model. Effective IgG-mediated phagocytosis targeting FcγR in mice is hampered by the lack of sufficient levels of naturally occurring anti–*S. aureus* Abs, as shown for several surface expressed and secreted proteins by multiplex assay (51) and proteome array (52). However, the purified proteins proved to be effective as an FcγR inhibitor in mice.

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**References**


