JLB

Article

Peptide length and folding state govern the capacity of staphylococcal β-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2

Dorothee Kretschmer,[*,1] *Maren Rautenberg,*[*] *Dirk Linke,*[†,2] *and Andreas Peschel*[*]

*Interfaculty Institute of Microbiology and Infection Medicine, Cellular and Molecular Microbiology Division, University of Tübingen, Germany; and †Max Planck Institute for Developmental Biology, Department I, Tübingen, Germany

RECEIVED MAY 30, 2014; REVISED DECEMBER 22, 2014; ACCEPTED JANUARY 19, 2015. DOI: 10.1189/jlb.2A0514-275R

ABSTRACT

Most staphylococci produce short α-type PSMs and about twice as long β-type PSMs that are potent leukocyte attractants and toxins. PSMs are usually secreted with the N-terminal formyl group but are only weak agonists for the leukocyte FPR1. Instead, the FPR1-related FPR2 senses PSMs efficiently and is crucial for leukocyte recruitment in infection. Which structural features distinguish FPR1 from FPR2 ligands has remained elusive. To analyze which peptide properties may govern the capacities of β-type PSMs to activate FPRs, full-length and truncated variants of such peptides from *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Staphylococcus lugdunensis* were synthesized. FPR2 activation was observed even for short N- or C-terminal β-type PSM variants once they were longer than 18 aa, and this activity increased with length. In contrast, the shortest tested peptides were potent FPR1 agonists, and this property declined with increasing peptide length. Whereas full-length β-type PSMs formed α-helices and exhibited no FPR1-specific activity, the truncated peptides had less-stable secondary structures, were weak agonists for FPR1, and required N-terminal formyl-methionine residues to be FPR2 agonists. Together, these data suggest that FPR1 and FPR2 have opposed ligand preferences. Short, flexible PSM structures may favor FPR1 but not FPR2 activation, whereas longer peptides with α-helical, amphipathic properties are strong FPR2 but only weak FPR1 agonists. These findings should help to unravel the ligand specificities of 2 critical human PRRs, and they may be important for new, anti-infective and anti-inflammatory strategies.
***J. Leukoc. Biol.* 97: 689–697; 2015.**

Abbreviations: AM = acetoxymethylester, CD spectroscopy = circular dichroism spectroscopy, FLIPr = formyl-peptide receptor 2-inhibitory protein, fMLF = *N*-formylmethionyl leucyl phenylalanine, FPR1–3 = formyl-peptide receptor 1–3, HBSS-HSA = HBSS containing 0.05% HSA, LDH = lactate dehydrogenase, PRR = pattern recognition receptor, PSM = phenol-soluble modulin, *Sa*PSMβ1 = *Staphylococcus aureus* PSMβ1,

(*continued on next page*)

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

The innate immune response is indispensable for the detection of invading microbial pathogens. Neutrophils are the first leukocytes infiltrating infected tissues. They become activated via PRRs, such as the TLRs or intracellular nucleotide-binding oligomerization domain receptors [1–3]. At early stages of infection, detection of bacteria occurs also via 7-transmembrane G-protein-coupled receptors, such as the FPRs [4–7]. Stimulation of these receptors leads to chemotactic migration of leukocytes, which distinguishes FPRs from other PRRs. A gradient of bacterial derived, formylated peptides, resulting from the bacteria-specific start of protein biosynthesis with a formylated methionine, guides neutrophils to the focus of infection [4]. The FPR1, sensing formylated peptides at very low concentrations, is an important innate immune receptor at the surface of various leukocytes, including neutrophils and monocytes [8]. Whereas FPR1 is dedicated to the sensing of short formylated peptides, such as fMLF, 2 paralogs (FPR2 and FPR3) have only very low affinity for formylated peptides and respond efficiently to other types of peptide agonists [6, 9, 10]. FPR2 senses a variety of endogenous peptides, such as the human antimicrobial peptide LL-37 [11]. In addition, FPR2 strongly responds to PSMs, cytolytic and proinflammatory peptides secreted by *S. aureus*, *S. epidermidis*, and most other staphylococcal species [12–15]. PSMs and other FPR2 ligands have only very limited sequence similarities and have been proposed to share a particular signature in their secondary structure [4, 6, 16, 17]. Notably, FPR1 responds with similar efficiency to most bacterial species, which all release formylated peptides, whereas FPR2 is specific for staphylococcal PSMs [13] and unknown peptides from enterococci [18] and *Listeria monocytogenes* [19]. As the virulence of staphylococci corresponds to the amounts of released PSMs, FPR2 can discriminate between highly and only weakly pathogenic staphylococcal strains [12]. Interestingly, although PSMs

1. Correspondence: Interfaculty Institute of Microbiology and Infection Medicine, Cellular and Molecular Microbiology Division, University of Tübingen, Elfriede-Aulhornstr. 6, 72076, Tübingen, Germany. E-mail: dorothee.kretschmer@med.uni-tuebingen.de

2. Current affiliation: Department of Biosciences, University of Oslo, Norway.

TABLE 1. Sequences of full-length and truncated peptides

Sample	Peptide	Sequence
A	SLUSH-B formylated	formyl-MSGIIEAITKAVQAGLDKDWATMGTSIAEALAKGIDAISGLFG
	SLUSH-B(N10) formylated	formyl-MSGIIEAITK
	SLUSH-B(N18) formylated	formyl-MSGIIEAITKAVQAGLDK
	SLUSH-B(N22) formylated	formyl-MSGIIEAITKAVQAGLDKDWAT
	SLUSH-B(N22)	MSGIIEAITKAVQAGLDKDWAT
	SLUSH-B(N26) formylated	formyl-MSGIIEAITKAVQAGLDKDWATMGTS
	SLUSH-B(C17)	IAEALAKGIDAISGLFG
	SLUSH-B(C21) formylated	formyl-MGTSIAEALAKGIDAISGLFG
	SLUSH-B(C21)	MGTSIAEALAKGIDAISGLFG
	SLUSH-B(C25)	DWATMGTSIAEALAKGIDAISGLFG
B	*Sa*PSMβ1 formylated	formyl-MEGLFNAIKDTVTAAINNDGAKLGTSIVSIVENGVGLLGKLFGF
	*Sa*PSMβ1(N22) formylated	formyl-MEGLFNAIKDTVTAAINNDGAK
C	*Se*PSMβ1 formylated	formyl-MSKLAEAIANTVKAAQDQDWTKLGTSIVDIVESGVSVLGKIFGF
	*Se*PSMβ1(N22) formylated	formyl-MSKLAEAIANTVKAAQDQDWTK
D	SLUSH-Brev formylated	formyl-GFLGSIADIGKALAEAISTGMTAWDKDLGAQVAKTIAEIIGSM
	fMLF	formyl-MLF

are secreted predominantly as formylated peptides, they activate FPR1 only very weakly [13, 20]

Staphylococci produce 2 types of PSM peptides: α-type and β-type PSMs, consisting of 20–26 or ~44 aa, respectively [12, 17, 21]. δ-Toxin represents 1 of the α-type PSMs [21]. All PSMs have α-helical, amphipathic structures [21]. In β-type PSMs, such as the *S. aureus* and *S. epidermidis* PSMβ1, as well as the SLUSH-B, only the C-terminal parts of the peptides have been assumed to be α-helical [17], but the actual secondary structures of β-type PSMs and the reasons why they are about twice as long as α-type PSMs have remained unknown. α-Type PSMs are, in general, more potent inducers of cell lysis and leukocyte activation than β-type PSMs [21–23]. However, β-type PSMs are usually secreted at higher concentrations than α-type PSMs, except δ-toxin, by different staphylococcal species [21, 24]. All PSMs, with the exception of the *S. aureus* PSM-mec, are core-genome encoded. The PSM-mec gene is located on a mobile genetic element [25]. It has been shown recently that PSMs are exported via the *S. aureus* ATP-binding cassette phenol-soluble modulin transporter (Pmt) that is essential for bacterial growth and contributes to producer immunity to secreted PSMs, which may exert antimicrobial activities at very high concentrations [26].

Staphylococci cause many types of infection. *S. aureus* is one of the most frequent causes of nosocomial and community-associated infections, including skin infections, abscesses, endocarditis, and sepsis [27]. In contrast to *S. aureus*, most coagulase-negative staphylococci have only a few virulence genes [28]. Many *S. aureus* strains produce chemotaxis inhibitory protein of *S. aureus*, FLIPr, and FLIPr-like, specific inhibitors of the human FPR1, FPR2, or both receptors, respectively [29–31]. These proteins are thought to enable the producing strains to remain unrecognized by the human innate immune defense during early stages of infection. The fact that *S. aureus* secretes these inhibitors underscores that FPRs are important receptors for the detection of bacteria and that disrupting these pathways may be a suitable strategy to modulate the activation of innate immune responses.

Here ,we analyzed whether β-type PSMs need their entire length for exerting their proinflammatory and cytolytic activities, and which part of the β-peptides is responsible for FPR2 activation. With the use of collections of β-type PSM congeners, we provide evidence that a peptide length of at least 18 aa, in combination with an N-terminal formylated methionine, favors FPR2 activation by PSMs, whereas a shorter sequence with a flexible folding state favors the capacity of formylated peptides to stimulate FPR1.

(*continued from previous page*)

*Se*PSMβ1 = *Staphylococcus epidermidis* PSMβ1, SLUSH-B = βPSM of *Staphylococcus lugdunensis*, SLUSH-Brev = synthethic SLUSH-B variant with reversed amino acid sequence

MATERIALS AND METHODS

Synthetic peptide and antibodies

S. lugdunensis and *S. epidermidis* PSM peptides were synthesized by EMC Microcollections (Tuebingen, Germany) and *S. aureus* PSMs by BACHEM (Bubendorf, Switzerland), with or without N-terminal formylation. The sequences of full-length and truncated peptides are given in Table 1. The SLUSH-B was also synthesized with reversed amino acid sequence for control studies, SLUSH-Brev. Antibodies against human FPR1 (mouse anti-human FPR1 MAB3744, isotype control MAB0031, and rat anti-mouse IgG2a-PE) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against human FPR2 were purchased from Genovac (Freiburg, Germany; mouse anti human FPR2, FN-1D6-A1) and from SouthernBiotech (Birmingham, AL, USA; goat anti-mouse IgG-R-PE).

Sequence analysis

Peptide helical-wheel calculations were accomplished with the HeliQuest tool (http://heliquest.ipmc.cnrs.fr) [32]. Sequence similarities were calculated with CLUSTALW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

Primary cells and cell lines

HL60 cells stably transfected with human FPR1 or FPR2 have been described recently [33, 34]. These cells were grown in RPMI medium (Biochrom, Cambridge, United Kingdom), supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO, USA), 20 mM HEPES (Biochrom), penicillin (100 units/ml), streptomycin (100 μg/ml; Gibco, Life Technologies, Grand

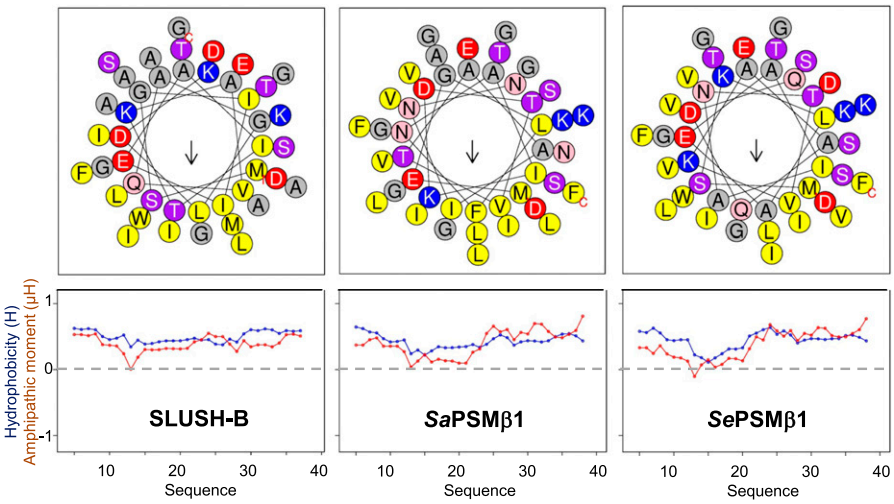

Figure 1. Helical wheel representation of SLUSH-B, *Sa*PSMβ1, and *Se*PSMβ1. (Upper) β-Type PSMs with amino acid properties shown in yellow (hydrophobic), red (negatively charged), blue (positively charged), purple or pink (polar but uncharged), and gray (small). (Lower) Mean hydrophobicity (H; blue line) and amphipathic moment (μH; red line) of β-type PSMs were calculated with the HeliQuest tool at a window size of 11 residues. The positive overall H and μH values indicate hydrophobic and amphipathic properties of the helical peptides, respectively.

Island, NY, USA), and 1× Glutamax (Gibco, Life Technologies). Transfected cells were grown in the presence of G418 (Biochrom) at a final concentration of 1 mg/ml.

Neutrophil chemotaxis

Human neutrophils were isolated by standard Ficoll/Histopaque gradient centrifugation. Chemotaxis toward synthetic peptides was determined by use of fluorescence-labeled neutrophils that migrated through a 3 μm pore-size polycarbonate Transwell filter, as described recently [13]. In brief, 5×10^6 neutrophils/ml were labeled with 3.3 μM bis-2-carboxyethyl-5-[and-6]-carboxyfluorescein-AM (Molecular Probes, Leiden, Netherlands) for 20 min at room temperature, washed, and resuspended in HBSS-HSA. The upper compartment of the Transwell system was filled with 100 μl-labeled neutrophils and placed into a well containing 600 μl HBSS-has, with or without chemoattractants (e.g., fMLF or β-type PSMs), and placed into a well. After incubation at 37°C under 5% CO_2 for 80 min, the inserts were removed, and the fluorescence of the wells was read in a fluorescence reader (FLUOstar OPTIMA; BMG Labtech, Ortenberg, Germany).The fluorescence measured was used to monitor migration after subtracting the background migration of HBSS-HSA. Peptides were used at concentrations of 50 nM, 500 nM, 1 μM, 5 μM, and 10 μM for β-type PSMs and 0.1 nM, 0.5 nM, 1 nM, 10 nM, 100 nM, and 1000 nM for fMLF. The measured relative fluorescence was corrected for buffer controls (buffer added only to lower compartment).

Measurement of calcium ion fluxes in human neutrophils and HL60 cells

Calcium fluxes were analyzed by stimulating cells loaded with Fluo-3-AM (Molecular Probes) and monitoring fluorescence with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), as described recently [13]. Synthetic chemoattractants were used at concentrations in the linear range of the dose-response curves. To stimulate HL60 cells, all peptides were used at the following concentrations: 10 nM, 100 nM, 250 nM, 500 nM, 1 μM, 2.5 μM, 5 μM, and 10 μM. To stimulate neutrophils, all peptides were used at the following concentrations: 50 nM, 500 nM, 1 μM, 2.5 μM, 5 μM, and 10 μM. Measurements of 2000 events were performed, and calcium flux was expressed as relative fluorescence corrected for buffer controls.

IL-8 production

Human IL-8 was measured by use of an ELISA kit (R&D Systems), according to the manufacturer's instructions. Neutrophils were incubated with indicated concentrations of the different peptides for 5 h at 37°C, 5% CO_2. Then, cells

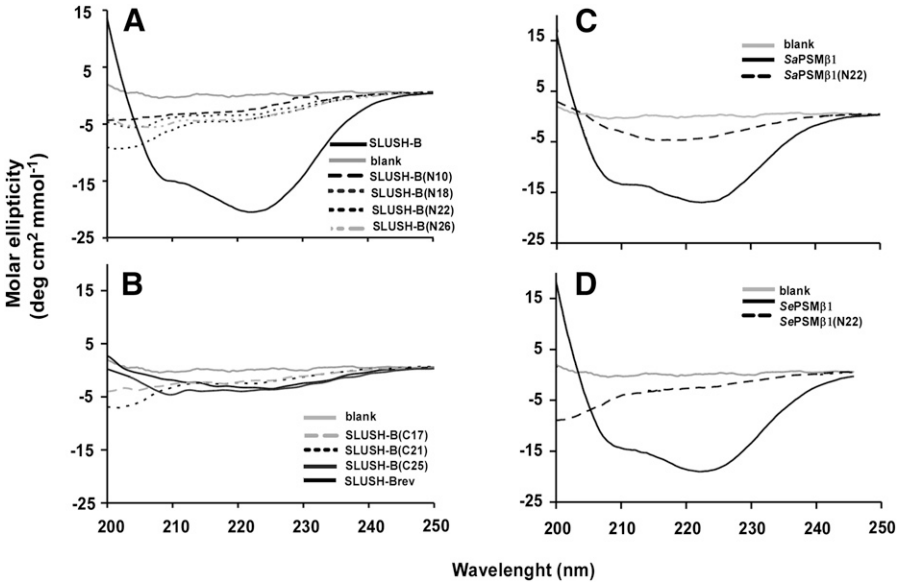

Figure 2. CD spectra of *Sa*PSMβ1, *Se*PSMβ1, SLUSH-B,and truncated variants. The strong CD signals of SLUSH B (A), as well as *Sa*PSMβ1 (C) *Se*PSMβ1 (D) between 205 and 225 nm with 2 distinct negative peaks are indicative of α-helical secondary structure, whereas the weaker signals of all other peptides (A–D) correspond to undefined secondary structures.

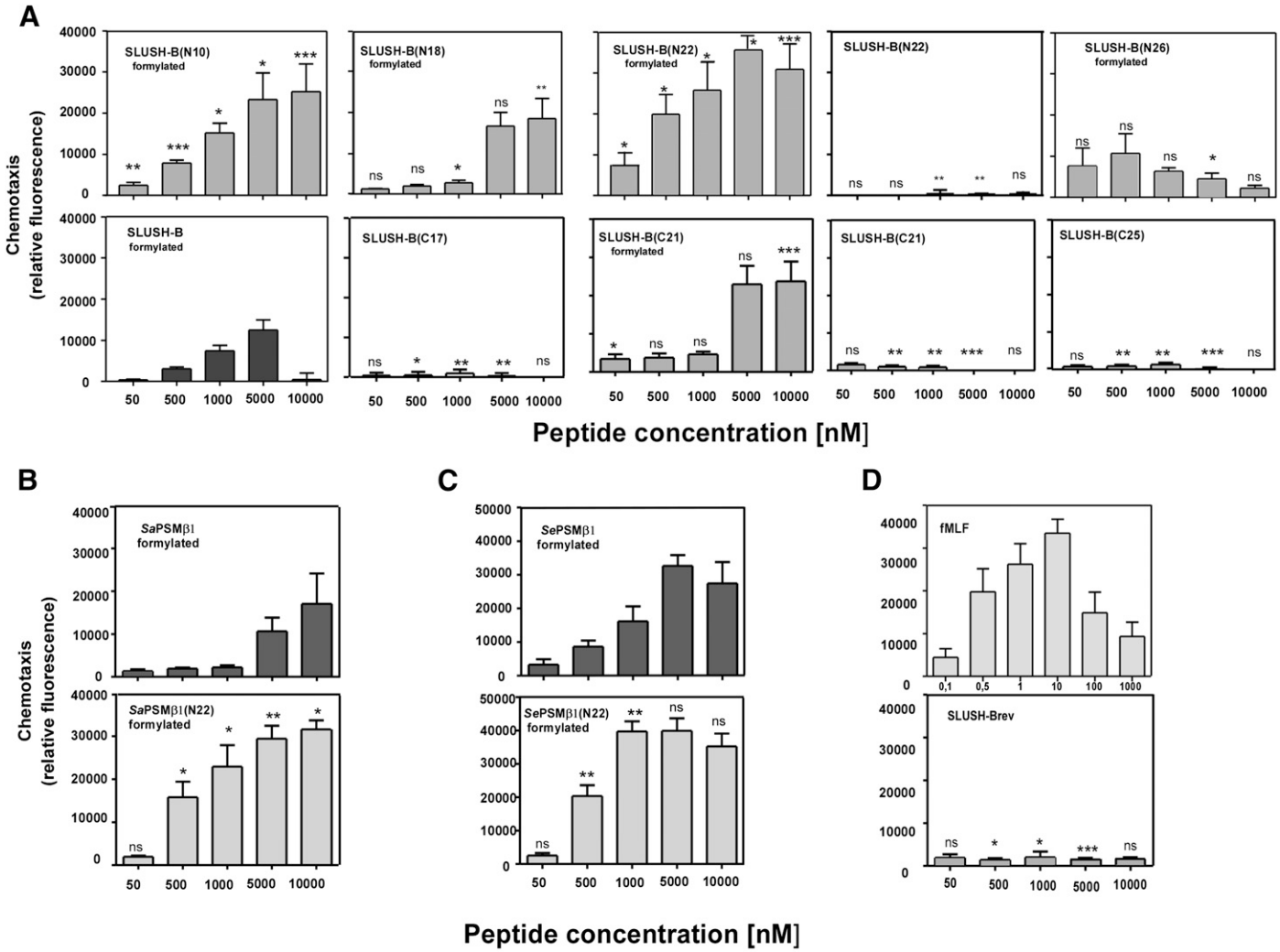

Figure 3. Chemotaxis induced by SLUSH-B, *Sa*PSMβ1, *Se*PSMβ1, and truncated variants. Neutrophil chemotaxis induced by increasing concentrations of full-length peptides or shortened variants of SLUSH-B (A), *Sa*PSMβ1 (B), or *Se*PSMβ1 (C) is shown. (D) Control peptides fMLF and SLUSH-Brev. Data represent mean ± SEM of 3 independent experiments with neutrophils from 3 different individuals. $^*P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$; ns, not significant (vs. native, full-length peptides).

were centrifuged for 10 min at 250 *g* and 4°C, and cell supernatants were stored at −20°C before use [21].

Cell lysis

Lysis of neutrophils by 8 μM full-length or truncated PSMs was determined by measuring the release of cytoplasmic LDH (Cytotoxicity Detection Kit; Roche Diagnostics, Indianapolis, IN, USA), as described recently [21].

CD11b up-regulation

Activation of human neutrophils was determined by measuring surface expression of CD11b. Neutrophils were incubated with 5 μM PSMs at 37°C with rotation for 60 min as described elsewhere [21]. Cells were stained with a PE-labeled antibody against CD11b (mAb 44; BD Biosciences) or isotype control antibody (BD Biosciences). Then, neutrophils were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

CD spectroscopy

CD spectra were measured by use of a Jasco J-810 instrument at room temperature. Cuvettes with a 0.1 cm path were used; 5 spectra were averaged at a scan speed of 50 nm/min, band width of 1 nm, and data pitch of 0.1 nm. Peptides were measured at a concentration of 50 μg/ml in water with 5% trifluoroethanol.

Statistical methods

Statistical analyses were performed with the Prism 4.0 package (GraphPad Software, La Jolla, CA, USA) and the between-group differences were analyzed for significance with the unpaired Student's *t*-test.

RESULTS

β-Type PSM length is critical for a stable secondary structure

β-Type PSMs are approximately twice as long as α-type PSMs [12, 17, 21], raising the question of whether they contain an additional domain or are simply extended versions of α-type PSMs. When we analyzed sequences of the representative β-type PSMs, PSMβ1 (*S. aureus* and *S. epidermidis*), and SLUSH-B, we noticed that their hydrophobic and amphipathic (distinct polar

and hydrophobic) properties are most pronounced in the C-terminal halves but extend over the entire length of the peptides (**Fig. 1**). *Sa*PSMβ1 is more related to *Se*PSMβ1 than to SLUSH-B over the entire peptide length (77.3% vs. 63.6% similarity, respectively; Supplemental Table 1).

To analyze if shorter fragments of β-type PSMs may have similar biologic activities as the full-length peptide synthetic SLUSH-B, congeners with stepwise truncation from the N and C terminus were generated. In addition, the N-terminal and C-terminal halves of *Sa*PSMβ1 and *Se*PSMβ1 were synthesized for functional studies (Table 1). The full-length and N-terminal peptides were generated as formylated peptides. As we noted that SLUSH-B bears a methionine at the junction between N- and C-terminal halves, in contrast to the other 2 β-type PSMs, the C-terminal SLUSH-B fragment SLUSH-B(C21) was generated in the formylated and nonformylated state to analyze whether formylation may affect the biologic activity of the C-terminal half of β-type PSMs. Likewise, the N-terminal SLUSH-B(N22) peptide was generated as a formylated and nonformylated peptide (Table 1).

As α-helical, amphipathic properties are assumed to be important for the capacity of PSMs to lyse neutrophils and activate FPR2 [17, 20] and as the propensity to form stable α-helices may depend on peptide length, the secondary structures of all 3 β-type PSMs and their subfragments were compared by CD spectroscopy (**Fig. 2**). Whereas all investigated, full-length β-type PSMs exhibited typical α-helical spectra, the truncated peptides behaved more like unfolded peptides, indicating that their secondary structure is less stable compared with the full-length peptides. A SLUSH-Brev did not exhibit an α-helical structure (Fig. 2B), indicating that the amino acid sequence is a crucial determinant for β-type PSM to form stable secondary structures.

Peptide length and formylation govern the capacities of β-type PSMs to attract, lyse, and activate human neutrophils

*Sa*PSMβ1, *Se*PSMβ1, and SLUSH-B have been shown to induce chemotactic migration of neutrophils [12, 21], raising the question of whether this property requires full-length peptides or may also be found in β-type PSM fragments. We found that all formylated N-terminal peptide fragments of the 3 β-type PSMs are chemoattractants for human neutrophils, and the shorter fragments were even stronger attractants than the full-length peptides (**Fig. 3A–C**). None of them was as active, though, as the most potent, known formylated peptide, fMLF (Fig. 3D). The nonformylated N-terminal SLUSH-B(N22) peptide did not induce migration of neutrophils (Fig. 3A). Notably, the formylated C-terminal fragment SLUSH-B(C21) also recruited neutrophils, but this activity was absent from the corresponding nonformylated peptide (Fig. 3A). The SLUSH-Brev peptide with a reversed amino acid sequence had no activity, probably as it neither bore a formylated methionine at its N terminus nor exhibited a stable secondary structure (Fig. 3D). Very similar results were obtained when neutrophil calcium fluxes were measured (Supplemental Fig. 1). Thus, β-type PSMs do not need their full length to attract neutrophils, but all shortened peptides require N-terminal formylation to exert this activity.

To investigate if the neutrophil-attracting properties of β-type PSM fragments correspond to their cytotoxic activity, the capacities of the various peptides to disrupt human neutrophils were compared. We found that full-length β-type PSMs have rather weak but notable cytolytic activities at micromolar concentrations. All 3 full-length β-type PSMs (8 μM) led to lysis of ~30% of neutrophils (**Fig. 4A–C**). In contrast, none of the N- and C-terminally shortened β-type PSM fragments had obvious cytolytic activities, indicating that it is possible to separate the cytolytic and chemoattractant ways of PSMs to affect leukocytes (Fig. 4A–C).

When the capacities of the 3 β-type PSMs to stimulate IL-8 release or CD11b up-regulation in human neutrophils were analyzed, *Sa*PSMβ1 was found to induce stronger CD11b up-regulation (**Fig. 5B**) and IL-8 secretion (Supplemental Fig. 2B) than SLUSH-B and *Se*PSMβ1 (Fig. 5A and C and Supplemental Fig. 2A and C). None of the nonformylated N-terminal or C-terminal fragments had such an activity. In contrast, all formylated N-terminal fragments and the formylated SLUSH-B (C21) induced CD11b up-regulation (Fig. 5A) and only slight [*Se*PSMβ1(N), *Sa*PSMβ1(N),] or no [SLUSH-B(N)] IL-8 secretion (Supplemental Fig. 3). Thus, the various β-type PSMs and truncated derivatives differ in their capacities to recruit, disrupt, and activate neutrophils, suggesting that the 3 activities may depend on different types on interactions.

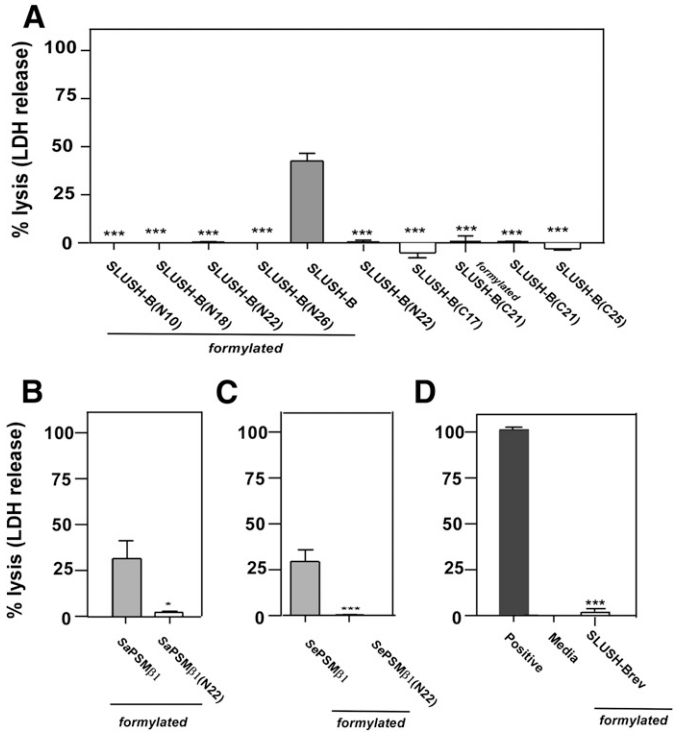

Figure 4. Lysis of neutrophils by SLUSH-B, *Sa*PSMβ1, *Se*PSMβ1, and truncated variants. Neutrophil lysis by 8 μM full-length peptides or shortened variants of SLUSH-B (A), *Sa*PSMβ1 (B), *Se*PSMβ1 (C), or SLUSH-Brev (D) is shown. 100%, Cell lysis mediated by 1% Triton X-100. Data represent means ± SEM of 3 independent experiments with neutrophils from 3 different donors. $*P < 0.05$; $***P < 0.001$ versus native, full-length peptides.

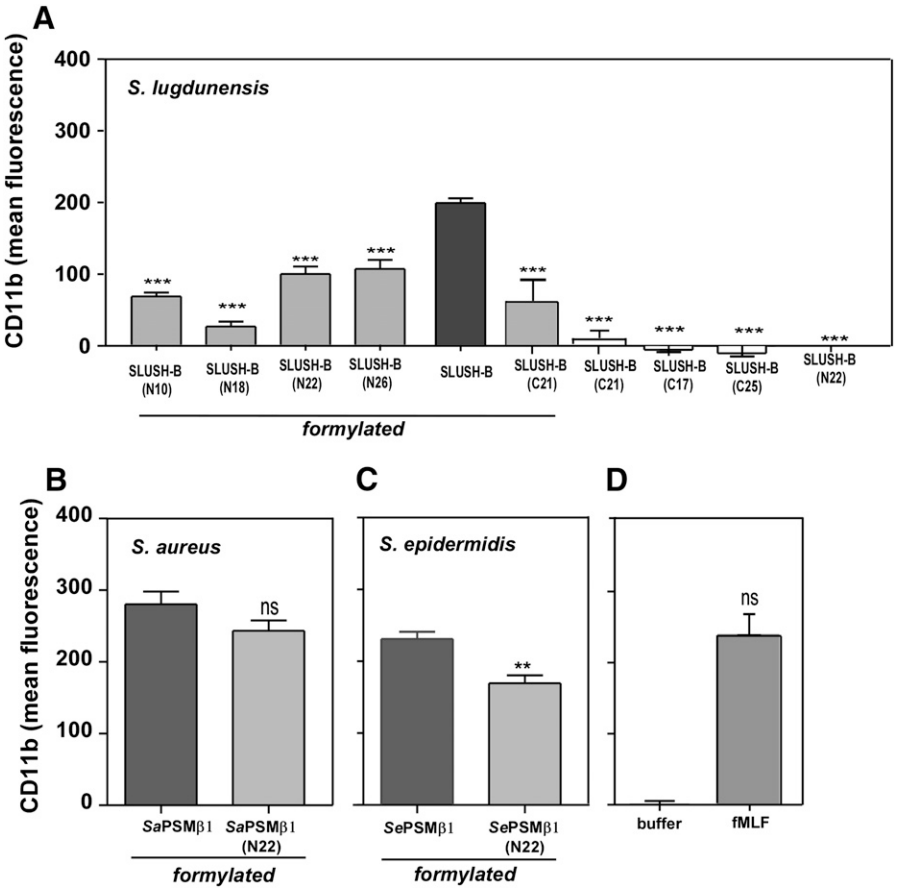

Figure 5. CD11b up-regulation on neutrophils by SLUSH-B, *Sa*PSMβ1, *Se*PSMβ1, and truncated variants. The CD11b-up-regulating capacities of 5 μM of the indicated β-type PSMs and truncated variants (A–C) or of 10 μM fMLF (D) are shown. Data represent means ± SEM of 3 independent experiments with neutrophils from 3 different individuals. $^{**}P < 0.01$; $^{***}P < 0.001$ (vs. native, full-length peptides).

FPR1 and FPR2 discriminate between β-type PSM variants based on peptide length and formylation

The various ways of β-type PSM fragments to modulate neutrophil functions raised the question of whether they can activate FPR2 or FPR1 in a similar way as the full-length peptides. HL60 cells, stably transfected with FPR1 or FPR2, and untransfected cells (Supplemental Fig. 4) [34] were stimulated with the various peptides, and calcium influx was measured as an established read-out for activation of FPRs [13].

The shortest N-terminal SLUSH-B fragment, SLUSH-B(N10), had no FPR2-stimulating but pronounced FPR1-stimulating activity (**Fig. 6A**). N-Terminal SLUSH-B fragments with lengths similar to those of α-type PSMs (18 aa or longer), however, had notable capacities to activate FPR2, which increased with length and reached the maximum in the 26 aa-long variant and in full-length SLUSH-B (Fig. 6A). Conversely, FPR1 activation declined with peptide length and was close to zero in full-length SLUSH-B (Fig. 6A). The N-terminal half of the *S. epidermidis* peptide *Se*PSMβ1(N22) (Fig. 6C) also activated FPR2 in a similar way as SLUSH-B(N22). However, *Sa*PSMβ1(N22) (Fig. 6B) differed from the 2 corresponding peptides, as it did not stimulate FPR2 but had substantial FPR1-stimulating activity. The absence of a formyl group in SLUSH-B(N22) abolished all activity to any of the 2 receptors (Fig. 6A). Thus, peptide length is a crucial parameter for the capacity of β-type PSM fragments to activate FPR2.

Notably, the formylated but not the nonformylated C-terminal half of SLUSH-B also had potent activity toward FPR2 and only weak activity to FPR1 (Fig. 6A). Together, these data indicate that the capacity of β-type PSMs to activate FPR2 does not require the full length of 42 aa but that shortened peptides can do so as long as they reach a certain length and bear an N-terminal formyl group.

DISCUSSION

FPR2 recognizes a growing list of virtually unrelated peptides, raising the question as to which molecular pattern may be recognized by this receptor [4, 35]. It has been shown previously that FPR1 recognizes a defined microbial molecular pattern, *N*-formylated methionine, followed by hydrophobic amino acids [36, 37], but it is still not clear which structural features define an FPR2 ligand [4, 6, 9, 10]. By truncating β-PSMs stepwise, we found that differences in PSM length and folding state have crucial impacts on the propensities of the peptides to stimulate FPR1 or FPR2. Reduced activation of FPR2 correlated in all 3 N-terminal peptides with increased FPR1 activation and vice versa, indicating that the 3 receptors have opposed ligand specificities. Short, formylated peptides are only very weak agonists for FPR2, although their activity increases with peptide length [20, 36, 38]. A recent study demonstrated that the shortening of α-type PSMs from the C terminus reduced their capacity to activate FPR2, which further corroborates the notion that peptide length is critical for FPR2 stimulation [20]. Nonformylated peptides with α-helical, amphipathic properties, such as human LL-37 [11]; the

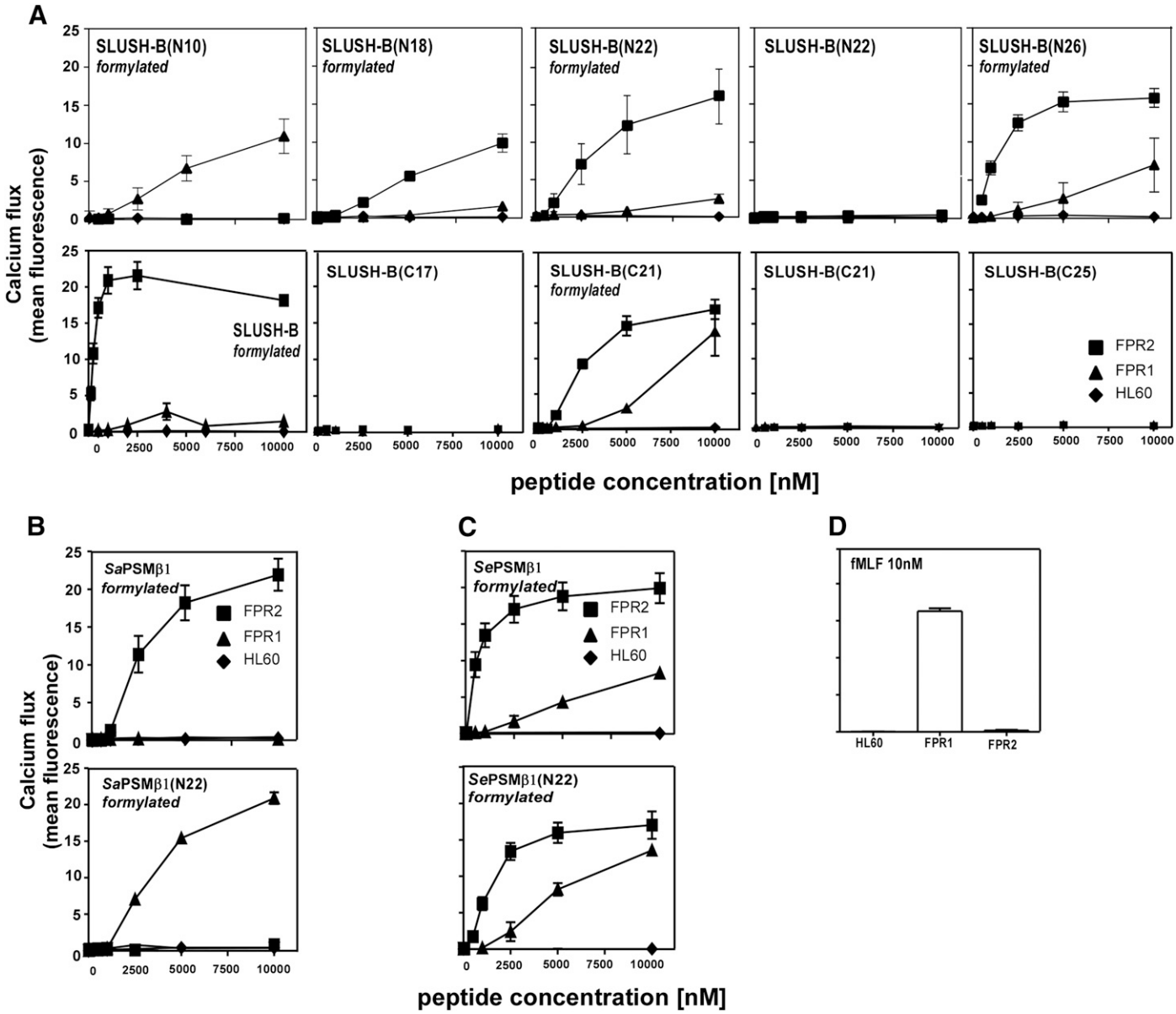

Figure 6. Stimulation of receptor-transfected HL60 cells by SLUSH-B, *Sa*PSMβ1, *Se*PSMβ1, and truncated variants. Calcium influx in receptor-transfected HL60 cells upon stimulation of full-length peptides or shortened variants of SLUSH B (A), *Sa*PSMβ1 (B), *Se*PSMβ1 (C), or fMLF (D) is shown. Data represent means ± SEM of at least 3 independent experiments.

Helicobacter pylori peptide Hp [2–20, 39]; or nonformylated, full-length α- of β-type PSMs [13] have intermediary capacities to activate FPR2, suggesting that such a secondary structure is a preferred motif for FPR2 [4]. The strongest, FPR2-specific activities have been reported for PSMs that combine N-terminal formylation and α-helical and amphipathic secondary structures [13]. At the same time, formylated, full-length PSMs are only very weak FPR1 agonists, suggesting that a stable secondary structure compromises their interaction with FPR1 [13].

The truncated, formylated β-type PSMs used in this study activated FPR2 only when they were 18 aa or longer. α-Type PSMs are in a similar size range and have documented α-helical structures, which is agreement with the assumption that such a secondary structure represents a crucial molecular pattern for FPR2 recognition. In contrast to the full-length β-type PSMs, the truncated peptides did not form stable α-helical structures in CD experiments. The stability of secondary structures is known to increase with peptide length, and many peptides adopt defined structures only when bound to a receptor protein [40]. With the consideration that CD experiments need to be carried out at rather artificial buffer conditions, it is tempting to assume that even the truncated β-type PSMs above 18 aa can form amphipathic α-helices in complex with FPR2 as a prerequisite for FPR2 activation. The fact that the capacity of all truncated β-type PSMs to activate FPR2 depended on N-terminal formylation may be a result of the instability of secondary structures, which may

require an initial interaction of the peptides with FPR2 via the formylated N terminus. On the other hand, the increased structural flexibility of the truncated β-type PSMs compared with native PSMs was reflected by the notable capacity of such peptides to activate FPR1. Similar observations were recently made with shortened α-type PSMs [20]. Thus, a stable α-helical and amphipathic structure appears to favor FPR2 but not FPR1 activation, whereas a shorter, undefined, flexible secondary structure may favor FPR1 but not FPR2 activation. It remains unclear why the N-terminal half of the *S. aureus* β-type PSM did not activate FPR2 at all. Its propensity to adopt a stable secondary structure may be even weaker than those of the corresponding parts of the *S. lugdunensis* and *S. epidermidis* peptides, which are more related to each other in terms of sequence than to *Sa*PSMβ1(N22) (Supplemental Table 1).

It is interesting to note that increased FPR1 compared with FPR2 activation by β-type PSMs and their subfragments was accompanied by stronger neutrophil chemotaxis and calcium influx, suggesting that FPR1 may be more abundant or may have a stronger capacity to elicit such responses in primary leukocytes than FPR2. The cytotoxic properties of β-type PSMs did not correspond to their receptor-activating capacities, as only the full-length β-type PSMs had such an activity. Likewise, the shortening of α-type PSMs leads to loss of cytotoxicity, whereas some FPR2-stimulating activity can still be observed [20]. It seems that very stable α-helical and amphipathic properties are required for the peptides to disrupt leukocyte membranes. FPR2 has been proposed to sense specifically the abundance of peptide toxins as a measure of pathogen virulence, whereas FPR1 detects bacterial formylated peptides in general [12]. The fact that reduced folding of PSMs simultaneously abrogates cytotoxicity and changes the capacity to stimulate FPR2 fits well with this concept and underscores the potential role of FPR2 as a receptor for true pathogen-associated molecular patterns. The patterns of IL-8 activation by the various peptides were somewhat intermediary between those observed for CD11b up-regulation and cytotoxicity, suggesting that both—receptor-dependent and membrane-damaging—activities may contribute to the release of IL-8 from intracellular neutrophil compartments [41].

It remains unclear why staphylococci produce β-type PSMs in addition to α-type PSMs. Notably, the N-terminal and C-terminal half of SLUSH-B could activate FPR2 as long as it was formylated. suggesting that β-type PSMs may have evolved from α-type PSMs by extension or duplication. Sequence alignments revealed that the β-type PSMs possess much higher similarity among PSMs of different species than α-type PSMs [12], suggesting that their primary sequence may have a more important functional role. β-Type PSMs are more abundantly secreted than α-type PSMs, except δ-toxin [21, 42], although their cytotoxic and proinflammatory activities are lower than those of α-type PSMs [21], suggesting that another function may be more important. In line with this notion, β-type PSMs play a more important role in biofilm detachment and structuring than α-type PSMs, which may be crucial in vivo [43]. We have found that secretion of high amounts of FPR2 ligands is typical for staphylococci, whereas many other bacteria, including *Escherichia coli* and *Yersinia enterocolitica*, secrete only FPR1 ligands [18]. How exactly FPRs recognize ligand peptides and if activation of the various FPRs has different consequences in leukocytes should be studied in the future. Such studies will help to develop new strategies to combat severe staphylococcal infections, e.g., by generating antagonists of FPR2 that would inhibit overwhelming inflammatory responses in acute infections induced by highly pathogenic *S. aureus*.

AUTHORSHIP

D.K., M.R., and A.P. conceived of and designed the experiments. D.K. and D.L. performed the experiments. D.K., D.L., and A.P. analyzed the data. D.K. and A.P. wrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the German Research Council (SFB685 and PE805/5-1, to A.P.; TRR34, to A.P. and D.K.; SFB766 to D.L.) and Fortüne Program of the Medical Faculty, University of Tübingen (to D.K.). The authors thank Michael Otto for helpful discussion and Nele Nikola, Cordula Gekeler, and Astrid Ursinus for excellent technical support.

DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1. Koizumi, Y., Toma, C., Higa, N., Nohara, T., Nakasone, N., Suzuki, T. (2012) Inflammasome activation via intracellular NLRs triggered by bacterial infection. *Cell. Microbiol.* **14,** 149–154.
2. Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P., Zychlinsky, A. (1999) Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* **285,** 736–739.
3. Fournier, B. (2012) The function of TLR2 during staphylococcal diseases. *Front. Cell. Infect. Microbiol.* **2,** 167.
4. Bloes, D. A., Kretschmer, D., Peschel, A. (2015) Enemy attraction: bacterial agonists for leukocyte chemotaxis receptors. *Nat. Rev. Microbiol.* **13,** 95–104.
5. Fu, H., Karlsson, J., Bylund, J., Movitz, C., Karlsson, A., Dahlgren, C. (2006) Ligand recognition and activation of formyl peptide receptors in neutrophils. *J. Leukoc. Biol.* **79,** 247–256.
6. Ye, R. D., Boulay, F., Wang, J. M., Dahlgren, C., Gerard, C., Parmentier, M., Serhan, C. N., Murphy, P. M. (2009) International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol. Rev.* **61,** 119–161.
7. Dürr, M. C., Kristian, S. A., Otto, M., Matteoli, G., Margolis, P. S., Trias, J., van Kessel, K. P., van Strijp, J. A., Bohn, E., Landmann, R., Peschel, A. (2006) Neutrophil chemotaxis by pathogen-associated molecular patterns—formylated peptides are crucial but not the sole neutrophil attractants produced by *Staphylococcus aureus*. *Cell. Microbiol.* **8,** 207–217.
8. Migeotte, I., Communi, D., Parmentier, M. (2006) Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine Growth Factor Rev.* **17,** 501–519.
9. Murphy, P. M., Ozçelik, T., Kenney, R. T., Tiffany, H. L., McDermott, D., Francke, U. (1992) A structural homologue of the N-formyl peptide receptor. Characterization and chromosome mapping of a peptide chemoattractant receptor family. *J. Biol. Chem.* **267,** 7637–7643.
10. Ye, R. D., Cavanagh, S. L., Quehenberger, O., Prossnitz, E. R., Cochrane, C. G. (1992) Isolation of a cDNA that encodes a novel granulocyte *N*-formyl peptide receptor. *Biochem. Biophys. Res. Commun.* **184,** 582–589.
11. De Yang, Chen, Q., Schmidt, A. P., Anderson, G. M., Wang, J. M., Wooters, J., Oppenheim, J. J., Chertov, O. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med.* **192,** 1069–1074.
12. Rautenberg, M., Joo, H. S., Otto, M., Peschel, A. (2011) Neutrophil responses to staphylococcal pathogens and commensals via the formyl

peptide receptor 2 relates to phenol-soluble modulin release and virulence. *FASEB J.* **25,** 1254–1263.
13. Kretschmer, D., Gleske, A. K., Rautenberg, M., Wang, R., Köberle, M., Bohn, E., Schöneberg, T., Rabiet, M. J., Boulay, F., Klebanoff, S. J., van Kessel, K. A., van Strijp, J. A., Otto, M., Peschel, A. (2010) Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. *Cell Host Microbe* **7,** 463–473.
14. Kretschmer, D., Nikola, N., Dürr, M., Otto, M., Peschel, A. (2012) The virulence regulator Agr controls the staphylococcal capacity to activate human neutrophils via the formyl peptide receptor 2. *J. Innate Immun.* **4,** 201–212.
15. Forsman, H., Christenson, K., Bylund, J., Dahlgren, C. (2012) Receptor-dependent and -independent immunomodulatory effects of phenol-soluble modulin peptides from *Staphylococcus aureus* on human neutrophils are abrogated through peptide inactivation by reactive oxygen species. *Infect. Immun.* **80,** 1987–1995.
16. Cheung, Y. C. G., Kretschmer, D., Queck, S. Y., Joo, H. S., Wang, R., Duong, A. C., Nguyen, T. H., Bach, T.-H. L., Porter, A. R., DeLeo, F. R., Peschel, A., Otto, M. (2014) Insight into structure-function relationship in phenol-soluble modulins using an alanine screen of the phenol-soluble modulin (PSM) alpha3 peptide. *FASEB J.* **28,** 153–161.
17. Peschel, A., Otto, M. (2013) Phenol-soluble modulins and staphylococcal infection. *Nat. Rev. Microbiol.* **11,** 667–673.
18. Bloes, D. A., Otto, M., Peschel, A., Kretschmer, D. (2012) *Enterococcus faecium* stimulates human neutrophils via the formyl-peptide receptor 2. *PLoS ONE* **7,** e39910.
19. Liu, M., Chen, K., Yoshimura, T., Liu, Y., Gong, W., Wang, A., Gao, J. L., Murphy, P. M., Wang, J. M. (2012) Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*. *Sci. Rep.* **2,** 786.
20. Forsman, H., Winther, M., Gabl, M., Skovbakke, S. L., Boulay, F., Rabiet, M. J., Dahlgren, C. (2015) Structural changes of the ligand and of the receptor alters the receptor preference for neutrophil activating peptides starting with a formylmethionyl group. *Biochim. Biophys. Acta* **1853,** 192–200.
21. Wang, R., Braughton, K. R., Kretschmer, D., Bach, T. H., Queck, S. Y., Li, M., Kennedy, A. D., Dorward, D. W., Klebanoff, S. J., Peschel, A., DeLeo, F. R., Otto, M. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat. Med.* **13,** 1510–1514.
22. Cheung, G. Y., Rigby, K., Wang, R., Queck, S. Y., Braughton, K. R., Whitney, A. R., Teintze, M., DeLeo, F. R., Otto, M. (2010) *Staphylococcus epidermidis* strategies to avoid killing by human neutrophils. *PLoS Pathog.* **6,** e1001133.
23. Giese, B., Glowinski, F., Paprotka, K., Dittmann, S., Steiner, T., Sinha, B., Fraunholz, M. J. (2011) Expression of δ-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of β-toxin. *Cell. Microbiol.* **13,** 316–329.
24. Vuong, C., Dürr, M., Carmody, A. B., Peschel, A., Klebanoff, S. J., Otto, M. (2004) Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell. Microbiol.* **6,** 753–759.
25. Queck, S. Y., Khan, B. A., Wang, R., Bach, T. H., Kretschmer, D., Chen, L., Kreiswirth, B. N., Peschel, A., Deleo, F. R., Otto, M. (2009) Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. *PLoS Pathog.* **5,** e1000533.
26. Chatterjee, S. S., Joo, H. S., Duong, A. C., Dieringer, T. D., Tan, V. Y., Song, Y., Fischer, E. R., Cheung, G. Y., Li, M., Otto, M. (2013) Essential *Staphylococcus aureus* toxin export system. *Nat. Med.* **19,** 364–367.
27. Lowy, F. D. (1998) *Staphylococcus aureus* infections. *N. Engl. J. Med.* **339,** 520–532.
28. Otto, M. (2009) *Staphylococcus epidermidis*—the 'accidental' pathogen. *Nat. Rev. Microbiol.* **7,** 555–567.
29. De Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P., van Strijp, J. A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J. Exp. Med.* **199,** 687–695.
30. Prat, C., Bestebroer, J., de Haas, C. J., van Strijp, J. A., van Kessel, K. P. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J. Immunol.* **177,** 8017–8026.
31. Prat, C., Haas, P. J., Bestebroer, J., de Haas, C. J., van Strijp, J. A., van Kessel, K. P. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from *Staphylococcus aureus* (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J. Immunol.* **183,** 6569–6578.
32. Gautier, R., Douguet, D., Antonny, B., Drin, G. (2008) HELIQUEST: a web server to screen sequences with specific alpha-helical properties. *Bioinformatics* **24,** 2101–2102.
33. Christophe, T., Karlsson, A., Dugave, C., Rabiet, M. J., Boulay, F., Dahlgren, C. (2001) The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J. Biol. Chem.* **276,** 21585–21593.
34. Dahlgren, C., Christophe, T., Boulay, F., Madianos, P. N., Rabiet, M. J., Karlsson, A. (2000) The synthetic chemoattractant Trp-Lys-Tyr-Met-Val-DMet activates neutrophils preferentially through the lipoxin A(4) receptor. *Blood* **95,** 1810–1818.
35. Schiffmann, E., Corcoran, B. A., Wahl, S. M. (1975) N-Formylmethionyl peptides as chemoattractants for leucocytes. *Proc. Natl. Acad. Sci. USA* **72,** 1059–1062.
36. Prossnitz, E. R., Quehenberger, O., Cochrane, C. G., Ye, R. D. (1993) Signal transducing properties of the N-formyl peptide receptor expressed in undifferentiated HL60 cells. *J. Immunol.* **151,** 5704–5715.
37. Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B., Becker, E. L. (1976) The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal secretion for neutrophils. *J. Exp. Med.* **143,** 1154–1169.
38. He, H. Q., Troksa, E. L., Caltabiano, G., Pardo, L., Ye, R. D. (2014) Structural determinants for the interaction of formyl peptide receptor 2 with peptide ligands. *J. Biol. Chem.* **289,** 2295–2306.
39. Bylund, J., Christophe, T., Boulay, F., Nyström, T., Karlsson, A., Dahlgren, C. (2001) Proinflammatory activity of a cecropin-like antibacterial peptide from *Helicobacter pylori*. *Antimicrob. Agents Chemother.* **45,** 1700–1704.
40. Raussens, V., Slupsky, C. M., Ryan, R. O., Sykes, B. D. (2002) NMR structure and dynamics of a receptor-active apolipoprotein E peptide. *J. Biol. Chem.* **277,** 29172–29180.
41. Pellmé, S., Mörgelin, M., Tapper, H., Mellqvist, U. H., Dahlgren, C., Karlsson, A. (2006) Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. *J. Leukoc. Biol.* **79,** 564–573.
42. Yao, Y., Sturdevant, D. E., Otto, M. (2005) Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J. Infect. Dis.* **191,** 289–298.
43. Wang, R., Khan, B. A., Cheung, G. Y., Bach, T. H., Jameson-Lee, M., Kong, K. F., Queck, S. Y., Otto, M. (2011) *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. *J. Clin. Invest.* **121,** 238–248.

KEY WORDS:
G-protein-coupled receptors · α-helical peptides · neutrophils