Constitutive and Inflammation-Dependent Antimicrobial Peptides Produced by Epithelium Are Differentially Processed and Inactivated by the Commensal *Finegoldia magna* and the Pathogen *Streptococcus pyogenes*

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Constitutive and Inflammation-Dependent Antimicrobial Peptides Produced by Epithelium Are Differentially Processed and Inactivated by the Commensal *Finegoldia magna* and the Pathogen *Streptococcus pyogenes*

Inga-Maria Frick,* Sara L. Nordin,† Maria Baumgarten,* Matthias Mörgelin,* Ole E. Sørensen,* Anders I. Olin,*† and Arne Egesten†

Epithelial linings serve as physical barriers and produce antimicrobial peptides (AMPs) to maintain host integrity. Examples are the bactericidal proteins midkine (MK) and BRAK/CXCL14 that are constitutively produced in the skin epidermal layer, where the anaerobic Gram-positive coccoid commensal *Finegoldia magna* resides. Consequently, this bacterium is likely to encounter both MK and BRAK/CXCL14, making these molecules possible threats to its habitat. In this study, we show that MK expression is upregulated during inflammation, concomitant with a strong downregulation of BRAK/CXCL14, resulting in changed antibacterial conditions. MK, BRAK/CXCL14, and the inflammation-dependent antimicrobial β-defensins human β-defensin (hBD)-2 and hBD-3 all showed bactericidal activity against both *F. magna* and the virulent pathogen *Streptococcus pyogenes* at similar concentrations. SufA, a released protease of *F. magna*, degraded MK and BRAK/CXCL14 but not hBD-2 nor hBD-3. Cleavage was seen at lysine and arginine residues, amino acids characteristic of AMPs. Intermediate SufA-degraded fragments of MK and BRAK/CXCL14 showed stronger bactericidal activity against *S. pyogenes* than *F. magna*, thus promoting survival of the latter. In contrast, the cysteine-protease SpeB of *S. pyogenes* rapidly degraded all AMPs investigated. The proteins FAF and SIC, released by *F. magna* and *S. pyogenes*, respectively, neutralized the antibacterial activity of MK and BRAK/CXCL14, protein FAF being the most efficient. Quantitation and colocalization by immunoelectron microscopy demonstrated significant levels and interactions of the molecules in in vivo and ex vivo samples. The findings reflect strategies used by a permanently residing commensal and a virulent pathogen, the latter operating during the limited time course of invasive disease. *The Journal of Immunology*, 2011, 187: 4300–4309.

Epithelial linings constitute a physical barrier against environmental microbes that present potential threats to the host (for instance, pathogenic bacteria) but are also colonized by bacteria that are part of the normal microbiota. In addition to being a physical barrier, epithelial cells have a constitutive production of antimicrobial agents; for example, antimicrobial peptides (AMPs) such as the antibacterial chemokine BRAK/CXCL14 (1–3). Another example is midkine (MK), an antibacterial growth factor that is constitutively expressed in the basal part of the epidermal layer (4, 5). It is likely that there is a delicate balance among constitutively produced AMPs, the ecological niche inhabited by bacteria of the normal microbiota, and the prevention of infection caused by pathogenic bacteria. In the case of injury and invasion of pathogenic bacteria, the expression of several AMPs of epithelial cells is induced, resulting in production of, for example, the β-defensins human β-defensin (hBD)-2 and hBD-3 (6, 7).

The normal human microbiota includes several bacterial species that inhabit the skin without causing any inflammation. An example of a skin commensal is the anaerobic Gram-positive coccus *Finegoldia magna*. This bacterium is also an opportunistic pathogen causing several clinical conditions, such as soft tissue infections, wound infections, and bone/joint infections in immunocompromised hosts (8). In addition, a number of recent studies reported the presence of *F. magna* in chronic wounds, including diabetic ulcers (9–11). *F. magna* resides in the lower parts of the epidermal layer, where it binds to BM-40, which is part of the basal membrane (BM), via the surface-associated protein FAF (12). It is therefore likely that *F. magna* encounter the constitutively expressed AMPs MK and BRAK/CXCL14 during normal, noninflamed conditions. Most strains of *F. magna* express a subtilisin-like enzyme, subtilase of *F. magna* (SufA), which is associated with the bacterial surface (13). Both FAF and SufA are produced at substantial amounts during early logarithmic phase, and, in addition to being surface associated, they are released to the environment (12, 13). Thus, it is likely that high local concentrations of these proteins can be reached in vivo. Studies on the proteolytic activity of SufA demonstrated that the enzyme cleaves...
and inactivates antibacterial molecules such as LL-37 and MIG/ CXCL9 (13). In addition, *F. magna* can neutralize the activity of AMPs by release of protein FAF from its surface (12, 14).

*Streptococcus pyogenes* is a highly virulent pathogen causing both superficial and deep severe infections, such as pharyngitis, erysipelas, necrotizing fasciitis, and septic shock (15). During infection and dissemination in tissues, *S. pyogenes* releases the cytotoxic protease SpeB that can degrade several molecules of the host response (16). In addition, *S. pyogenes* releases protein SIC that inhibits complement activation and neutralizes AMPs (17–21). Both SpeB and SIC are produced in high amounts during early growth phase (20, 22), and both proteins are important for the virulence of *S. pyogenes* (22–27).

In this study, we show that the commensal *F. magna* can circumvent the bactericidal activity of MK, BRAK/CXCL14, and hBD-3 by releasing SufA and protein SIC. The findings explain how *F. magna* can create a protected ecological niche during noninflamed conditions, whereas *S. pyogenes* possesses potent means to avoid a broad range of host defense molecules produced in response to inflammation during invasive infection. These properties are likely to reflect different strategies used by a permanently colonizing commensal and a highly virulent pathogen, the latter operating during the limited time course of invasive disease.

**Materials and Methods**

**Proteins and peptides**

Native SufA and SpeB were purified as previously described (13, 28). Recombinantly expressed protein FAF (aa 28–616) was obtained as a fusion protein with GST as described (12). Human recombinant MK and BRAK/CXCL14 were from PeproTech (Rocky Hill, NJ). hBD-2 and hBD-3 were from Creative Peptides (Shirley, NY).

**Human skin samples**

Samples from human skin wounds were obtained under protocols approved by the Ethical Committee at Lund University (LU 509-01 and LU 708-01). Nonwounded human skin was obtained by taking punch biopsies from healthy donors, and skin wound samples were obtained by making new punch biopsies from the edges of the initial biopsies. Parts of the samples were fixed in formalin (10%) for immunohistochemistry. For cDNA microarray analysis, as much dermal tissue as possible was removed by subcutaneous dissection from the biopsies. The remaining tissue was washed thoroughly in sterile sodium chloride (0.9%) to remove infiltrating inflammatory cells. The specimens were incubated at room temperature for 1 h with polyclonal antibodies to *S. pyogenes* (Lund, Sweden).

**Immunohistochemistry for light microscopy**

After fixation in formalin (10%), the specimens were dehydrated, embedded in paraffin, placed on Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany), incubated for 2 h at 60°C, deparaffinized in Tissue Clear (Sakura Finetek, Zoeterwoude, The Netherlands), and rehydrated in graded alcohol and water. The slides were then treated with Dako Ag Retrieval Solution (Dako/Cytomation, Glostrup, Denmark) for 40 min at 97°C, followed by blocking in TBS supplemented with 0.05% Tween 20, 1% BSA, for 45 min at room temperature. After three 15-min washings, the specimens were incubated at room temperature for 1 h with polyclonal Abs diluted (5 μg/ml) in TBS supplemented with 0.05% Tween 20, 1% BSA. After three 20-min washings in TBS with 0.05% Tween 20, the slides were incubated with a MACH 3 Probe alkaline phosphatase Polymer kit (Biocare Medical, Concord, CA) for 10 plus 10 min, followed by three 15-min washes. Color was developed using Vector Fast Red chromogen (Biocare Medical, Concord, CA), and the slides were counterstained with Harris Hematoxylin (Histolab, Göteborg, Sweden).

**Cell culture and stimulation of cells**

Primary human keratinocytes were obtained from Cascade Biologics (Portland, OR) and cultured in serum-free medium (KGM2 Bullet kit) from Cambrex (Karlskoga, Sweden). Cells were stimulated, beginning 24 h after complete confluence was reached with conditioned medium from PBMCs that had been stimulated with LPS (100 ng/ml *Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, MO) for 4 d as described (27). After stimulation of the keratinocytes for 48 h, the cells were solubilized in TRIzol (Invitrogen, Carlsbad, CA) and RNA purified according to the instructions from the manufacturer.

**RNA isolation and microarray analysis**

Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according to the recommendations of the supplier and resuspended in 0.1 mM EDTA. The concentration was determined by spectrophotometric measurement.

For gene expression analysis, total RNA was biotinylated and hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix, Santa Clara, CA) according to the instructions by the manufacturer. The microarray fluorescence signals were normalized using the GeneChip Operation Software (GOS ver. 1.4; Affymetrix). The analysis was performed essentially as previously described (30). To calculate fold change in mRNA expression, the cDNA hybridization signals for MK and BRAK/CXCL14 obtained from wounds on day 4 were compared with the signals obtained from nonwounded microarray. Data are available through the Minimum Information About a Microarray Experiment database (http://www.ebi.ac.uk/arrayexpress; accession number E-MEXP-3305; user name: Reviewer, E-MEXP-3305; password: 18dxiehx).

**Quantitative real-time RT-PCR**

cDNA was synthesized from 400 ng purified RNA from the keratinocyte culture using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the instructions given by the manufacturer. The expression of MK and BRAK/CXCL14 together with G3PDH was determined using iQ SYB Green Supermix (Bio-Rad). Primers for real-time PCR were: MK forward, 5-GCT TCC TCC TCC TCA CCC TC-3; reverse, 5-TTA TCT TCT TTT TTG GCG ACC G-3; and BRAK/CXCL14 forward, 5-CTC AAG TTT GGT TGC CAG AA-3; and reverse, 5-GAA ATC CTC AGG TGG GAA AA-3. Amplification was performed at 55°C for 40 cycles in an iCycler Thermal Cycler (Bio-Rad) and data analyzed using iCycler iQ Optical System Software (Bio-Rad). To calculate fold change in mRNA levels, the threshold cycles (Ct) of MK and BRAK/CXCL14, respectively, were subtracted from the Ct of the *G3PDH* housekeeping gene, thus obtaining normalized Ct. To calculate the difference in mRNA levels for MK and BRAK/CXCL14, values of resting keratinocytes were subtracted from those of stimulated keratinocytes and expressed as ΔΔCt. Negative value of ΔΔCt denotes downregulation, and positive ΔΔCt denotes upregulation.

**Bacterial strains and growth conditions**

*F. magna* strains 505 and ALB8 were isolated at Lund University Hospital (Lund, Sweden). *S. pyogenes* strain API (4058) of serotype M1 was from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. API was grown in Todd–Hewitt broth (TH; BD/Difco, Franklin Lakes, NJ) at 37°C with 5% CO2. *F. magna* bacteria were grown in TH supplemented with 0.5% Tween-80 at 37°C under strict anaerobic conditions (Anaerobic Workstation; Elektrotek, Shipley, U.K.).

**Bactericidal assay**

*F. magna* bacteria (505 strain) were cultivated for 24 h (exponential growth phase, OD600 ≈ 0.4), washed in incubation buffer (10 mM Tris/ HCl, 5 mM glucose [pH 7.5]), and adjusted to a concentration of 2 × 1010 CFU/ml. Fifty microliters bacterial solution (~108 CFU) was incubated in buffer or with various concentrations of MK, BRAK/CXCL14, hBD-2, or hBD-3 for 1 h under strict anaerobic conditions. To quantify the bactericidal activity, serial dilutions of the mixtures were plated on TH-agar (supplemented with Tween-80) in duplicates and incubated at 37°C for 3 d under anaerobic conditions. The number of CFUs was then determined.
mRNA levels (ddCt) in normalized Ct (dCt) (calculated by subtracting the
termined by quantitative RT-PCR. The chart depicts the difference in
from PBMC for 48 h. MK and BRAK/CXCL14 expression were de-
Primary keratinocytes were stimulated with LPS-conditioned medium
expression, whereas BRAK/CXCL14 was downregulated (top panel).

Cleavage of AMPs with SufA and SpeB

Digestion of MK, BRAK/CXCL14, hBD-2, or hBD-3 with SufA and SpeB
was performed at 37°C for 1 and 18 h, respectively. For cleavage with
SufA, 5 μg peptides were incubated with 0.5 μg enzyme in PBS. The
reactions were terminated by the addition of PMSF (Sigma-Aldrich) to
a final concentration of 5 mM. In the case of SpeB, 5 μg peptides were
incubated with 0.5 μg enzyme in PBS supplemented with DTT (final
concentration 10 mM). The reactions were terminated by the addition of
the cysteine-protease inhibitor E64 (final concentration 10 mM).
The cleaved peptides were separated and analyzed by Tricine SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli using a total poly-
acrylamide concentration of 10% and 3.3% cross-linking. Analysis of
peptide degradation was performed with Tricine SDS-PAGE (31) using
a polyacrylamide concentration of 16.5% and 3.3% cross-linking. Gels
were stained with Coomassie R-250.

Mass spectrometry

Obtained Coomassie-stained gel protein bands after SufA digestion were
selected, cut, washed with 40% (v/v) acetonitrile, and evaporated to dryness.
Reduction was performed with 10 mM DTT at 36°C for 30 min, alkylation
with 30 mM iodoacetamide, and digestion with Asp-N endopeptidase
(Roche Diagnostics, Mannheim, Germany; 25 μg/ml) in 25 mM ammo-
nium bicarbonate buffer (pH 7.8) for 16 h at 37°C. After extraction of gel
pieces with 75% acetonitrile in 5% trifluoroacetic acid, peptides were
subjected to reversed-phase HPLC separation (CapLC; Waters, Man-
chester, U.K.) and subsequent sequencing on a Q-TOF Ultima API MS/MS
(Waters, Manchester, U.K.). MK and BRAK/CXCL14 incubated with
SufA were also taken directly to liquid chromatography tandem mass
spectrometry (MS/MS) for analysis of obtained shorter peptide products.
The MS/MS results were interpreted using the Peptide Mass Fingerprint
tool of the Mascot search engine (http://www.matrixscience.com). Settings
of fixed and variable modifications were used. Experimental data were
compared with Asp-N cleavage of the MK and BRAK/CXCL14 sequences

Transmission electron microscopy

For negative staining, bacteria were incubated with intact AMPs or pro-
teolytic fragments as described above, followed by processing for trans-
mission electron microscopy as previously described (32). In short, samples
(5 μl) were absorbed for 2 min onto the grids followed by two washes with
distilled water. The samples were stained for 3 s with 0.75% uranyl for-
mate droplets, followed by staining for an additional 30 s with 0.75%
uranyl formate.

For immunolabeling, fixed and washed samples were subsequently
dehydrated in ethanol and further processed for epon embedding. Sections
were cut with an LKB ultratome (LKB, Bromma, Sweden) and mounted on
gold grids. For immunostaining, the grids were floated on top of drops of
immune reagents displayed on a sheet of parafilm. For inactivation of
glutaraldehyde and osmium tetroxide used during fixation, saturated so-
dium metaperiodate was applied, and for elimination of unspecific binding,
the grids were then incubated with 50 mM glycine, and 5% (v/v) goat
serum in incubation buffer (0.2% Aurion-BSA [Aurion] in PBS [pH 7.6]).
This blocking procedure was followed by overnight incubation with
polyclonal rabbit Abs: anti-SufA, anti-FAF, anti-SpeB, anti-SIC, and

FIGURE 1. MK and BRAK/CXCL14 expression in skin. A, Bound
specific Abs against MK and BRAK/CXCL14, respectively, were detected by
secondary alkaline phosphatase-conjugated Abs, reacting with a sub-
strate that is deposited as a red stain. MK and BRAK/CXCL14 are detected
in epidermis of normal skin. MK shows a stronger staining in the basal
parts, whereas BRAK/CXCL14 is more evenly distributed (top panel). In
skin from an acute wound (day 4; bottom panel), dispersed expression of
MK is seen in the epidermal layer, whereas BRAK/CXCL14 expression is
decreased. Replacement of the specific Abs with rabbit control IgG
resulted in loss of staining. B, Electron micrograph showing overview with
basal cell at the epidermal/dermal junction (stratum germinativum, aster-
isk; top left panel) and prickle cell in stratum spinosum in the epidermis of
healthy skin. Scale bar, 5 μm. Subcellular localization of MK and BRAK/
CXCL14 in keratinocytes was determined using immunogold where bound
specific Abs were detected using secondary Abs conjugated with colloidal
gold (10 nm). Both MK and BRAK/CXCL14 are seen in association with
the BM (lamina densa; asterisk), at the nuclear membrane (NM), and in
association with the plasma membrane (PM) in keratinocytes of healthy
skin, whereas only weak labeling for BRAK/CXCL14 was seen in kera-
tinocytes of skin wounds after 4 d. Scale bar, 1 μm. C, To compare MK
and BRAK/CXCL14 gene expression, mRNA was isolated from epidermis of
healthy skin and of epidermis obtained by biopsy at the wound edges
4 d postinjury. cDNA microarray revealed an ~3-fold upregulation of MK
gene expression, whereas BRAK/CXCL14 was downregulated (left panel).
Primary keratinocytes were stimulated with LPS-conditioned medium
from PBMC for 48 h. MK and BRAK/CXCL14 expression were de-
termined by quantitative RT-PCR. The chart depicts the difference in
mRNA levels (ddCt) in normalized Ct (dCt) (calculated by subtracting the

S. pyogenes (API strain) was cultivated to midexponential growth phase
(OD600 ≈ 0.4). The bacteria were washed, diluted in incubation buffer, and
~10⁶ CFU were incubated in buffer or with various concentrations of MK,
BRAK/CXCL14, hBD-2, or hBD-3 for 1 h at 37°C. The mixtures were
diluted, plated on TH-agar, incubated at 37°C overnight, and the number of
CFUs were counted. All dilutions were performed in incubation buffer.
Bacteria were also incubated with SufA- and SpeB-degraded peptides for 1
h at 37°C, and the bactericidal activity was quantified as described above.

MK and BRAK/CXCL14 Ct from the Ct of the G3PDH housekeeping gene
that was used as reference) between stimulated wells compared with
nonstimulated controls. A negative ddCT value depicts downregulation and
a positive value upregulation. A weak upregulation of MK gene expression
was found, whereas BRAK/CXCL14 was downregulated by ~12 threshold
cycles (right panel).
polycyclonal anti-MK, and anti-BRAK/CXCL14 at 4˚C. Signal for
binding was detected with EM goat anti-rabbit IgG 10 nm Gold (BBI-
International, Cardiff, U.K.) and EM rabbit anti-goat IgG 20 nm Gold
(BBInternational), respectively. Samples were finally stained with uranyl
acetate and lead citrate before being observed and evaluated as above.
They were examined with a Jeol JEM 1230 EX transmission electron
microscope (Jeol) operated at 60 kV accelerating voltage. Images were
recorded with a Gatan Multiscan 791 charge-coupled device camera
(Gatan, Pleasanton, CA).
To evaluate the concentrations of the AMPs and the bacterial proteins in
skin, the gold probe density on specific primary Abs and on normal rabbit
Ig controls was quantified by taking 30 random electron micrographs on
individual skin samples. Micrographs were masked with overlays repre-
senting 1 μm². The number of gold particles within different 1-μm² win-
dows was determined manually by point counting in each micrograph.
Molar Ag concentrations were then calculated on characteristics of Ag
retention procedures (33–35) and predictions on immunolabeling effi-
ciency, surface relief of thin sections, and surface accessibility of randomly
dispersed molecules as described (36).

Surface plasmon resonance spectrometry
Protein FAF, protein SIC, or hBD-3 was immobilized via amine coupling
in flow cells of a CM5 sensorchip (BIAcore, Uppsala, Sweden). The
immobilization levels were ~1000 resonance units. A flow cell subjected to
the coupling reaction without added protein was used as a control for bulk
refraction index changes. For affinity measurements, binding and disso-
ciation were monitored in a BIAcore 2000 instrument (BIAcore). Different
concentrations of proteins were injected over the coated surface at 10 μl
min and 25˚C (in running buffer: 10 mM HEPES [pH 7.5], 150 mM NaCl,
flow cell of each injected concentration were subtracted. The association
After X and Y normalization of data, the blank curves from the control
ning buffer containing 2 M NaCl followed by an extensive wash procedure.
the obtained rate constants.

KD

librium dissociation constants (3).

Results
Expression of MK and BRAK/CXCL14 in the epidermis during
healthy and inflamed conditions
Using immunohistochemistry, both MK and BRAK/CXCL14 could be detected in the epidermis of skin obtained from
healthy donors (Fig. 1A). Labeling for MK was most intense in
the basal parts of epidermis. However, in biopsies from wound
edges 4 d postinjury, a more dispersed staining for MK was ob-
served, whereas the staining for BRAK/CXCL14 was less intense,
suggesting a reciprocal gene regulation for these genes during
inflammation.
Subcellular localization of MK and BRAK/CXCL14 in kerati-
cocytes was determined using immunoelectron microscopy. Both
MK and BRAK/CXCL14 are seen in association with the BM
(lamina densa; asterisks, Fig. 1B), at the nuclear membrane, and in
association with the plasma membrane in keratinocytes of healthy
skin, whereas only weak labeling for BRAK/CXCL14 was seen in
keratinocytes of skin wounds after 4 d (Fig. 1B).
Using cDNA microarray, an ∼3-fold increase in MK expression
was seen 4 d after injury, whereas BRAK/CXCL14 was down-
regulated (Fig. 1C). To investigate the influence of inflammatory
mediators on MK and BRAK/CXCL14 expression in vitro, PBMC
were stimulated with LPS (100 ng/ml) for 4 d. The conditioned
medium was then used to stimulate primary human keratinocytes
for 48 h. The conditioned medium doubled the MK expression,
whereas BRAK/CXCL14 expression was downregulated by 12
threshold cycles (Fig. 1C). Downregulation of BRAK/CXCL14
expression in inflammatory skin disorders has been described
previously (3).

Bactericidal activity of MK, BRAK/CXCL14, hBD-2, and
hBD-3 against F. magna and S. pyogenes
Previous studies have demonstrated that MK and BRAK/CXCL14
both possess antibacterial activity (3, 5). Having seen the strong
upregulation of MK and the downregulation of BRAK/CXCL14
during inflammation, we therefore investigated whether the ac-
tivity of the proteins differ depending on the bacterial species being a commensal or a pathogen. In these experiments, we
compared the sensitivity of the commensal F. magna (strain 505)
and the pathogenic S. pyogenes (strain AP1) in a viable count
assay. Interestingly, MK was more potent in killing S. pyogenes
bacteria, whereas BRAK/CXCL14 killed F. magna bacteria more
efficiently (Fig. 2A, 2B). Thus, at a MK concentration of 0.1 μM,
~100% of S. pyogenes bacteria were killed, whereas 1 μM of the
peptide was required to achieve 100% killing of F. magna bac-
teria. In contrast, 0.1 μM BRAK/CXCL14 killed ~80% of F.

magnabacteria, but only 20% of the S. pyogenes bacteria. During
infection and inflammation, the β-defensins hBD-2 and hBD-3 are
upregulated and secreted by keratinocytes, and the bactericidal
activity of these peptides was also compared against F. magna
(strain 505) and S. pyogenes (strain AP1). Of these peptides, hBD-
3 was the most potent and efficiently killed both strains at 1 μM
(Fig. 2D). At this concentration, hBD-2 only slightly affected F.
magnabacteria (Fig. 2C) and, to achieve 100% killing of both F.
magna and S. pyogenes bacteria with this peptide, concentrations
of 10 μM were required.

Proteolytic activity from SufA of F. magna and SpeB of S.
pyogenes against MK, BRAK/CXCL14, hBD-2, and hBD-3 and
characterization of residual bactericidal activity

SufA of F. magna has proteolytic activity against LL-37 and
the chemokine MIG/CXCL9 (13). Also, SpeB of S. pyogenes
is known to cleave LL-37 (37) and several chemokines (38). Thus,
we incubated MK, BRAK/CXCL14, hBD-2, and hBD-3 with
SufA and SpeB, respectively, for 1 and 18 h, and the resulting
fragments were analyzed by Tricine SDS-PAGE (Fig. 3). SpeB

FIGURE 2. Bactericidal activity of MK (A), BRAK/CXCL14 (B), hBD-
2 (C), and hBD-3 (D) against F. magna and S. pyogenes. Bacteria were
grown to midlogarithmic phase and incubated with peptides at the in-
dicated concentrations for 1 h. To determine bactericidal activity, the
number of CFUs was determined and compared with that obtained after
incubation in buffer alone. The data shown represent mean ± SEM of three
to five separate experiments.
rapidly cleaved all polypeptides, and after 1 h of incubation, they were almost completely degraded (Fig. 3A–D). In contrast, SufA cleavage of MK generated several smaller fragments, and two stable fragments were observed over time (Fig. 3A), whereas BRAK/CXCL14 was completely degraded after 18 h of incubation (Fig. 3B). To identify the cleavage sites in MK and BRAK/CXCL14, the protein bands, indicated in Fig. 3A and 3B, were excised and subjected to MS/MS analysis. In addition, cleaved peptides in solution were also analyzed by MS/MS. The predicted SufA cleavage sites are indicated in the amino acid sequences of MK and BRAK/CXCL14 (Fig. 3E, 3F). Furthermore, in the models of these molecules, the amino acid residues prone to cleavage are indicated (Fig. 3E, 3F). The bactericidal activity of MK has been located to a region in the N-terminal domain and to the C-terminal tail (5). As shown in Fig. 3E, several cleavage sites for SufA are identified in these regions. Cationic amino acids (i.e., arginine and lysine) are characteristic features of AMPs, and the majority of cleavage sites included either of these amino acids. SufA did not degrade the defensins hBD-2 and hBD-3, neither as seen after SDS-PAGE (Fig. 3C, 3D) nor as determined by MS/MS.

Next, the bactericidal activity of the SufA-generated fragments of MK and BRAK/CXCL14 was tested to compare the activity against *F. magna* (505 strain) and *S. pyogenes* (AP1 strain). The fragments generated after 1 h of digestion with SufA were still able to efficiently kill *S. pyogenes*, but killed *F. magna* to a significantly lesser degree. The bactericidal activity was lost following further digestion (Fig. 4A, 4B). Electron microscopy revealed rupture of bacterial membranes and leakage of intracellular contents in the presence of the intact polypeptides, but to a lesser extent in the case of *F. magna*, when incubated with SufA-cleaved MK and BRAK/CXCL14, respectively (Fig. 4C). This suggests that the activity of SufA against these constitutively expressed AMPs will provide survival advantages for *F. magna*, at least during healthy conditions and during early stages of infection.

**FIGURE 3.** Proteolytic activity of SufA and SpeB, respectively, against MK (A), BRAK/CXCL14 (B), hBD-2 (C), and hBD-3 (D). The peptides (5 µg) were incubated with the respective enzymes (0.5 µg) for 1 and 18 h at 37˚C, and cleavage products were analyzed by Tricine SDS-PAGE. The fragments of MK and BRAK/CXCL14 generated by SufA after 1 h were characterized by MS/MS. The primary amino acid sequences and models of MK and BRAK/CXCL14 with predicted SufA cleavage sites indicated in red are shown in E and F, respectively. No cleavage of hBD-2 or hBD-3 with SufA occurred as determined by MS/MS.
Neutralizing capacity from protein FAF of *F. magna* and protein SIC of *S. pyogenes* against MK and BRAK/CXCL14

Most strains of *F. magna* express the surface protein FAF, which also is released into the surrounding environment, partly through action of SufA (12, 14). Thus, we asked whether FAF could bind and inhibit the activity of the AMPs described above. First, possible interference of FAF with the killing of *F. magna* bacteria by MK and BRAK/CXCL14 was investigated. The *F. magna* 505 strain was incubated with bactericidal concentrations of MK (1 μM), BRAK/CXCL14 (1 μM), and hBD-3 (1 μM), respectively, in presence of various ratios of FAF. As shown in Fig. 5A, FAF dose dependently blocked the activity of all three peptides. Notably, the defensin hBD-3, which was resistant to cleavage by SufA, was the peptide most efficiently blocked by FAF. To investigate binding characteristics between FAF and the various peptides, surface plasmon resonance spectrometry was used. FAF was immobilized to the surface of a chip, and MK, BRAK/CXCL14, or hBD-3 were injected and left to interact to saturation. Binding to FAF was obtained with MK (K_D = 13 nM) and BRAK/CXCL14 (K_D = 8.9 nM), but not hBD-3. However, when, instead, hBD-3 was immobilized and FAF injected over the surface, a stable interaction was observed (K_D = 0.7 nM) (Fig. 5A). Due to the relatively low and sometimes inconsistent bactericidal activity of hBD-2 in viable counts experiments, blocking experiments were not performed with this peptide. In addition, surface plasmon resonance experiments showed no or low binding of FAF to hBD-2 (data not shown).

Then we analyzed whether protein SIC, which is produced by some strains of *S. pyogenes* including the highly virulent M1 serotype (21), is also able to inhibit the bactericidal activity of MK and BRAK/CXCL14. SIC blocked the killing of *S. pyogenes* with these peptides, although not as efficiently as protein FAF (Fig. 5B). Furthermore, using surface plasmon resonance spectrometry, SIC was found to bind to both MK and BRAK/CXCL14, but with a more transient kinetic compared with the interaction of FAF (Fig. 5B). SIC has previously been shown to bind and interfere with the activity of human β-defensins and was therefore not analyzed (19, 39).

Colocalization of MK and BRAK/CXCL14 with SufA and FAF of *F. magna* and SpeB and SIC of *S. pyogenes* in infested skin

Healthy skin infested ex vivo with *F. magna* and biopsies from *S. pyogenes*-infected skin (erysipelas) were investigated using electron microscopy (Fig. 6A, upper panels). In both cases, bacteria were seen in the vicinity of keratinocytes, with characteristic morphological features (i.e., being coccoid and, in the case of *S. pyogenes*, an extracellular rim of M protein).

Using immunoelectron microscopy on healthy skin biopsies infested with *F. magna* ex vivo, MK and BRAK/CXCL14 were colocalized with SufA and FAF in close proximity of *F. magna* bacteria (Fig. 6A). In skin biopsies from erysipelas (caused by *S. pyogenes*), MK and BRAK/CXCL14 colocalized with SpeB and SIC, respectively, close to the bacterial surface (Fig. 6A). To determine the concentrations of the AMPs and the bacterial proteins in the skin samples, the gold probe density on the specific primary Abs and on normal rabbit Ig controls was quantified as described in Materials and Methods. The values obtained (Fig. 6B) confirmed increased production of MK and a decreased production of BRAK/CXCL14 during infection. Skin infected ex vivo with *F. magna* may resemble the situation during an opportunistic infection causing inflammation rather than colonization as a commensal. The findings are summarized in Fig. 7.

Discussion

In this study, we show that *F. magna*, a member of the normal microbiota, and the virulent pathogen *S. pyogenes* differently modulate the activity of constitutively and induced AMPs, respectively. Although *F. magna* possess the means (i.e., SufA and protein FAF) to circumvent the constant pressure of the constitutively expressed AMPs MK and BRAK/CXCL14, *S. pyogenes* possess a broader activity (via SpeB and protein SIC) including the important AMPs hBD-2 and hBD-3 that are expressed during inflammation (summarized in Figure 7).

During healthy, noninflamed conditions, *F. magna* colonize the basal parts of epidermis and are likely to encounter the
constitutively expressed MK and BRAK/CXCL14, as demonstrated in the current study. However, during inflammation, BRAK/CXCL14 production is downregulated, as seen in atopic dermatitis and psoriasis (3, 40). Epidermal growth factor is upregulated during inflammation and wound healing and has a strong inhibitory activity on BRAK/CXCL14 gene expression (41). MK is upregulated during inflammation in some tissues of the adult and can be detected in plasma of healthy individuals (42, 43). Binding of many proinflammatory cytokines to their corresponding receptors and activation of TLRs by pathogen-associated molecular patterns result in activation of NF-κB–dependent gene transcription (44). MK has an NF-κB–responsive element in its promoter region and is thus likely to be upregulated during inflammation (45). Another property of MK is that it recruits and activates neutrophils to sites of inflammation, suggesting several important roles for this growth factor (46).

In a previous study, it was shown that BRAK/CXCL14 has antibacterial activity against fungi and both Gram-positive and -negative aerobic bacteria (3). The finding in the present work that BRAK/CXCL14 had higher activity against F. magna than S. pyogenes might reflect this chemokine’s role in controlling the host burden of commensal bacteria. During inflammation caused by more virulent bacteria, additional AMPs such as hBD-2 and hBD-3 are upregulated to restrict the spread of the infection (47). Thus, in such an event, it is necessary for commensal bacteria to cope with another set of host defense molecules to survive. It is therefore not surprising that F. magna is less sensitive to MK. Interestingly, hBD-2 showed relatively weak antibacterial activity against both F. magna and S. pyogenes compared with the other AMPs investigated. During invasion, S. pyogenes, being a virulent pathogen, operates during a limited timeframe. This may be one explanation for SpeB being more efficient and less specific than...
SufA, enhancing successful invasion and dissemination, overwhelming the host. In contrast, it is not in the interest of *F. magna*, being a colonizing commensal, to disturb homeostasis. Common characteristics for AMPs are a high content of the positively charged amino acids arginine and lysine. Interestingly, SufA preferentially cleaves both MK and BRAK/CXCL14 at these positions and thereby reduces the antibacterial activity of these molecules. For a commensal bacterium, this is an important mechanism that will lead to improved survival, unarming constitutively produced AMPs, at the site of the colonization. The preference of SufA to cleave at positions of basic amino acids has been described for other subtilases of human and plant origin (48). In contrast, SufA cannot cope with the β-defensins, possibly because they are more compact molecules. However, *F. magna* strains that express FAF have an advantage through the efficient neutralizing capacity of this molecule against hBD-3. The peptide hBD-2 has a relatively low bactericidal activity and may not present a similar threat to the bacterium.

In the case of a pathogen, such as *S. pyogenes*, it is important for the bacterium to modulate a broad spectrum of AMPs to

![FIGURE 6. Colocalization of MK and BRAK/CXCL14 with SufA and FAF of *F. magna* and SpeB and SIC of *S. pyogenes* in infested skin. A. Healthy skin biopsies, ex vivo infested with *F. magna* (left panels) and biopsies from *S. pyogenes*-infected skin (erysipelas; right panels), were investigated using electron microscopy. Bacteria are seen in the vicinity of keratinocytes (top left panel, arrows). Scale bars, 2.5 μm. At higher magnification, individual bacteria are visualized. In the case of *S. pyogenes*, a typical rim of M protein is seen (upper right panel). Scale bars, 0.5 μm. Immunoelectron microscopy shows colocalization of MK (as detected by 10-nm colloidal gold particles conjugated with secondary Abs) and BRAK/CXCL14 (10-nm colloidal gold particles) with SufA (5-nm colloidal gold particles) and FAF (5-nm colloidal gold particles), respectively, in the close proximity of *F. magna* bacteria (bottom left panels). Scale bar, 0.1 μm. In a skin biopsy from erysipelas (caused by *S. pyogenes*), MK (as visualized by Abs labeled with 10-nm colloidal gold particles) and BRAK/CXCL14 (10-nm colloidal gold particles) colocalize with SpeB (5-nm colloidal gold particles) and SIC (5-nm colloidal gold particles), respectively, close to the bacterial surface (bottom right panels). Scale bar, 0.1 μm. B. Morphometric evaluation of AMPs and bacterial protein concentrations in skin. The probe density of gold-labeled Abs was quantified on electron micrographs of individual skin samples. Molar Ag concentrations were calculated based on the number of gold particles per μm².

![FIGURE 7. Schematic illustration of bacterial survival strategies during noninflamed and inflamed conditions, respectively. *F. magna* resides in the basal parts of epidermis, where it adheres to BM-40 of the BM. Keratinocytes constitutively produce MK and BRAK/CXCL14, for which bactericidal activity *F. magna* can attain by the release of SufA and FAF. Upon invasion of the virulent pathogen *S. pyogenes*, the host responds with inflammation, resulting in downregulation of BRAK/CXCL14 expression and a concomitant upregulation of MK, hBD-2, and hBD-3. *S. pyogenes* can neutralize these AMPs by release of the cysteine protease SpeB and the AMP-binding protein SIC, whereas the countermeasures executed by *F. magna* are less efficient in the inflamed context, because SufA has no effect on hBD-2 and hBD-3.]
efficiently invade host tissues. In this study, we find that SpeB rapidly degrades all investigated AMPs (MK, BRAK/CXCL14, hBD-2, and hBD-3). This is in line with previous studies showing a broad capacity for SpeB to cleave AMPs (38).

In the case of F. magna, we are not aware of other enzymes or proteins interacting with AMPs, whereas S. pyogenes produce several additional enzymes counteracting innate and adaptive immune mechanisms (e.g., SpyCep [degrading IL-8/CXCL8], C5a-peptidase [degrading C5a], IdeS [degrading IgG], and EndoG [glycanase activity against IgG]). However, the latter enzymes have a very selective mode of action (49).

The release of protein FAF and protein SIC by F. magna and S. pyogenes, respectively, equips the bacteria with means to inactivate AMPs at a distance. Interestingly, FAF was proven to bind and neutralize the antibacterial activity of both MK and BRAK/CXCL14 more efficiently than SIC. The survival of F. magna bacteria is critically dependent on balancing the pressure from constitutive host-defense activities, limiting its proliferative activity and virulence, whereas virulent pathogens such as S. pyogenes bacteria do not have a biology that needs such considerations.

Taken together, the current study demonstrates how F. magna can counteract the activities of constitutively produced AMPs, thus creating a protected habitat within the epidermis.

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Disclosures
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