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Antimicrobial peptides derived from the cartilage.-specific C-type Lectin Domain Family 3 Member A (CLEC3A) – potential in the prevention and treatment of septic arthritis

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SUMMARY

Objective: To investigate the antimicrobial activity of peptides derived from C-type Lectin Domain Family 3 Member A (CLEC3A), shed light on the mechanism of antimicrobial activity and assess their potential application in prevention and treatment of septic arthritis.
Design: We performed immunoblot to detect CLEC3A peptides in human cartilage extracts. To investigate their antimicrobial activity, we designed peptides and recombinantly expressed CLEC3A domains and used them to perform viable count assays using *E.coli*, *P.aeruginosa* and *S.aureus*. We investigated the mechanism of their antimicrobial activity by fluorescence and scanning electron microscopy, performed ELISA-style immunoassays and transmission electron microscopy to test for lipopolysaccharide binding and surface plasmon resonance to test for lipoteichoic acid (LTA) binding. We coated CLEC3A peptides on titanium, a commonly used prosthetic material, and performed fluorescence microscopy to quantify bacterial adhesion. Moreover, we assessed the peptides' cytotoxicity against primary human chondrocytes using MTT cell viability assays.
Results: CLEC3A fragments were detected in human cartilage extracts. Moreover, bacterial supernatants lead to fragmentation of recombinant and cartilage-derived CLEC3A. CLEC3A-derived peptides killed *E.coli*, *P.aeruginosa* and *S.aureus*, permeabilized bacterial membranes and bound lipopolysaccharide and LTA. Coating CLEC3A antimicrobial peptides (AMPs) on titanium lead to significantly reduced bacterial adhesion to the material. In addition, microbicidal concentrations of CLEC3A peptides *in vitro* displayed no direct cytotoxicity against primary human chondrocytes.
Conclusions: We identify cartilage-specific AMPs originating from CLEC3A, resolve the mechanism of their antimicrobial activity and point to a novel approach in the prevention and treatment of septic arthritis using potent, non-toxic, AMPs.

Introduction

Millions of arthroplasties are performed every year.[1] An estimated 1–2% of these procedures lead to septic arthritis.[2] Septic arthritis can lead to irreversible loss of joint function and shows high mortality rates.[3] In case of an infection, the dense cartilage extracellular matrix limits the ability of immune cells to infiltrate

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the tissue.[4] This explains why an alternative defense mechanism evolved in cartilage, namely, the production of antimicrobial peptides (AMPs).[5] AMPs are highly conserved, short (15–50) amino acid sequences with potent antimicrobial activity.[6,7] Research on AMPs in cartilage, however, is scarce, and only a few ubiquitous AMPs have been described in articular cartilage.[3,5,8,9]

In light of the expression of ubiquitous AMPs in cartilage, the question arises whether cartilage-specific AMPs exist. This could be the case with the C-type lectin domain family 3 member A (CLEC3A).[10] CLEC3A is a 23-kDa protein closely related to tetranectin,[11] another member of group IX of the C-type lectin family. On the protein level, CLEC3A is cartilage-specific and can be found in articular cartilage as well as in resting, proliferating, and hypertrophic growth-plate cartilage. Moreover, CLEC3A can bind plasminogen and contributes to its activation.[10] CLEC3A shows several features of an AMP precursor. First, it is highly susceptible to proteolytic cleavage,[12] which represents the first step in releasing active AMPs, as in the case of cathelicidin[13] and anti-thrombin III.[14] Second, an N-terminal fragment of CLEC3A exhibits strong heparin-binding activity, a feature frequently observed in AMPs.[14–16] Finally, another member of the C-type lectin family, the human Reg3A, has been shown to kill bacteria by forming membrane-permeabilizing pores.[17,18]

We therefore performed an exploratory study to examine whether cartilage-specific CLEC3A and CLEC3A-derived peptides exhibit antimicrobial activity, investigated their mechanism of action and assessed CLEC3A-derived peptides' potential in preventing arthroplasty-associated septic arthritis.

Method

Ethics

The study was approved by the ethics committee of the University Hospital Cologne (application no. 04–196).

Synthetic peptides

CLEC3A-derived peptides and TiBP (titanium-binding peptide) were synthesized by Biomatik (Canada) or Peptide 2.0 (VA, USA). LL-37 was purchased from Invivogen (CA, USA). The purity of the peptides was >98%.

Recombinant proteins

Full-length CLEC3A, the carbohydrate-recognition domain (CRD) and the oligomerization domain (OD) encoded by exon 2 and exon 3 (CLEC3A-Ex23) and only the CRD encoded by exon 3 (CLEC3A-Ex3) were recombinantly expressed and affinity purified as previously described[10] (Fig. 1).

Microorganisms

Viable count assays, fluorescence microscopy and bacterial cleavage experiments were performed using the following bacterial strains: *Escherichia coli (E.coli)* (ATCC 29522), *Pseudomonas aeruginosa* (*P.aeruginosa*) (ATCC 27853), *Staphylococcus aureus* (*S.aureus*) (ATCC 29213). Electron microscopy experiments were performed using the following bacterial strains: *Streptococcus pyogenes* (*S.pyogenes*) AP1 (40/58) of serotype M from the World Health Organization Collaborating Centre for Reference and Research on *Streptococci*, Prague, Czech Republic, *S.aureus* 111 and *E.coli* B1351, collected at the Department of Clinical Microbiology, Lund University Hospital, Sweden and *P.aeruginosa* PAO1 (ATCC, Teddington Oly, UK), originally isolated from a wound. Experiments on titanium were performed using *E.coli* GFP (ATCC 25922GFP).

Viable count assay

Bacteria were grown to mid-logarithmic phase ($OD_{630} \approx 0.5$) in tryptic soy broth (TSB) (Sigma–Aldrich, Germany). The viable count assay was performed as previously described.[14] Briefly, bacteria were washed and resuspended with Tris–HCl/Glucose (TG-buffer) (10 mM Tris–HCl 5 mM Glucose, pH 6.5) to a final concentration of 10^6 cfu/ml. Bacteria were incubated with water (negative control), 10μM of LL-37 (CA, USA) or synthetic peptide/recombinant protein solution for 2 h. Serial dilutions of the mixtures were plated out on TSB agar plates. The number of grown colonies in each mixture is displayed as a percentage of colonies grown in the control. Results are displayed as averages from three to four independent experiments and the standard deviation was calculated for each displayed value.

Fluorescein isothiocyanate (FITC) uptake assay

Bacteria were grown to mid-logarithmic phase ($OD_{630} \approx 0.5$) in luria bertani (LB) medium (Sigma–Aldrich, Germany). Bacteria were washed and resuspended in tris glucose buffer (TG-buffer) to a final concentration of $5x10^7$ cfu/ml. They were incubated with 20μM peptide solutions or water (negative control) for 2 h, and the assay was performed by using F7250 fluorescein isothiocyanate (FITC) (Sigma–Aldrich, Germany) as previously described.[14] Bacteria were visualized using a confocal laser scanning microscope (Leica TCS SP8). The displayed images show representative results from at least three independent experiments.

Scanning electron microscopy

Bacteria were grown to mid-logarithmic phase in Todd-Hewitt broth (THB) (DI, USA). They were washed and resuspended with Tris–HCl, pH 7.4 to a concentration of $2x10^9$ cfu/ml and were incubated for 2 h at 37°C with 2μM CLEC3A-derived peptides, 3μM LL-37 or Tris–HCl alone (negative control). Bacteria were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 (cacodylate buffer), washed with cacodylate buffer and dehydrated with ascending ethanol concentrations as previously described.[19] Samples were visualized with a Philips/FEI XL30 field emission guns (FEG) scanning electron microscope. The displayed images show representative results from at least three independent experiments.

Transmission electron microscopy

The binding of synthetic peptides to lipopolysaccharide (LPS), or to the bacterial surface of *E.coli*, was visualized by negative staining and transmission electron microscopy as previously described.[20] Briefly, LPS (5 μg/ml), or bacteria (2×10^9 cfu/ml), were incubated with synthetic peptides for 1 h at 37°C. For visualization in the electron microscope, the peptides were conjugated with 5 nm colloidal gold.[21] For visualization of LPS particles on bacterial membranes, a monoclonal antibody against *E.coli* LPS (ab35654) (Abcam, United Kingdom) was used conjugated with 10 nm colloidal gold. Specimens were examined in a Philips/FEICM 100 Twin transmission electron microscope. Images were recorded using a side-mounted Olympus Veleta camera with the ITEM acquisitions software. The displayed images show representative results from at least three independent experiments.

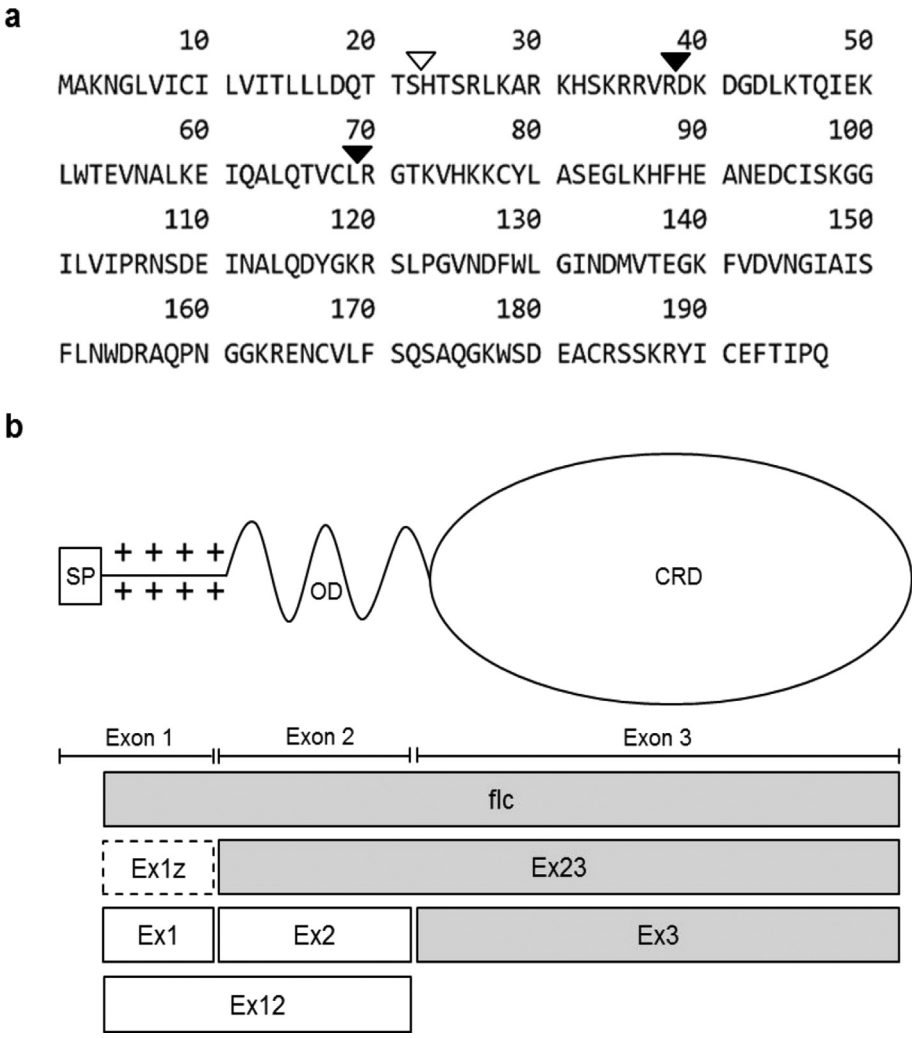

Fig. 1. Amino acid sequence and gene structure of CLEC3A with a schematic depiction of recombinant proteins and synthetic peptides. (a) CLEC3A consists of 197 amino acids. Open arrowhead marks the signal peptide cleavage site. Closed arrowheads mark the exon borders. (b) Schematic illustration of CLEC3A's gene structure and the exons encoding the protein, which comprise of a signal peptide and a positively charged sequence encoded by exon 1, a potential alpha-helical oligomerization domain (OD) encoded by exon 2, and a carbohydrate-recognition domain (CRD) encoded by exon 3. Grey rectangles represent the recombinant proteins; the white rectangles represent the synthetic peptides used in this study. The dotted line rectangle represents the synthetic peptide Ex1z, which contains the amino acid sequence of Ex1 without the positive charge. Recombinant full-length CLEC3A is abbreviated as flc.

LPS-binding assay

LPS-binding assay was performed in trademark of thermo fisher scientific (NUNC™) Maxi Sorb 96-well plates (Thermo Fisher Scientific, USA) by coating them overnight with 0.5 μg of synthetic peptide, recombinant protein, or LPS in sodium hydrogen phosphate, pH 9. Samples were blocked for 2 h at room temperature (RT) with 2% bovine serum albumin in PBS-T (VWR Chemicals, PA, USA) (blocking buffer) and incubated for 1 h at RT with 0.5 μg of Biotin-labeled LPS (Invivogen, CA, USA) or 0.5 μg of recombinant proteins. Samples incubated with Biotin-labeled LPS were subsequently incubated for 1 h at RT with a 1:2500 solution of streptavidin-peroxidase S5512 (Sigma–Aldrich, Germany) in blocking buffer. Samples incubated with the recombinant proteins were incubated for 1 h at RT with a 1:1000 solution of an affinity-purified rabbit anti-human CLEC3A antibody,[10] and subsequently for 1 h at RT with a 1:2500 solution of a swine anti-rabbit IgG HRP-labeled antibody (Dako, Denmark). All samples were developed by using the 3,3′,5,5′-tetramethylbenzidine liquid substrate system for enzyme-linked immunosorbent assay (ELISA) (Sigma–Aldrich, Germany). Absorption values were measured at 450 nm. Results are averages from three independent experiments, which were conducted in triplicate. The standard deviation was calculated for each displayed value.

Surface plasmon resonance

Surface plasmon resonance was performed as described previously[22] using a BIAcore2000 system (Sweden). Briefly, lipoteichoic acid (LTA) from *S.aureus* (L2515, Sigma–Aldrich, Germany) was covalently coupled to a CM-5 sensor chip (GE Healthcare, Germany) at 500 response units in 10 mM sodium acetate buffer, pH 5. To investigate binding to LTA, 300 nM of CLEC3A peptides and domains in HBS-EP buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 0.15 M NaCl, 3 mM ethylenediamine tetraacetic acid (EDTA), 0.005% (v/v) surfactant were passed over the LTA-coupled sensor chip. Experiments were performed in duplicate.

Tissue extraction

Human hip cartilage was obtained from a patient who underwent joint replacement surgery due to coxarthrosis. Extraction of the cartilage was performed by crushing the tissue in liquid

nitrogen as previously described.[10] Proteins were extracted overnight at 4°C with four volumes (v/w) of 2 M urea, 10 mM EDTA, 1 M NaCl, 50 mM Tris–HCl, pH 7.4.

Bacterial cleavage experiments

P.aeruginosa were grown overnight to mid-logarithmic phase ($OD_{630} \approx 0.5$) in TSB (Sigma–Aldrich, Germany). Bacteria were washed with dulbecco's phosphate buffered saline (DPBS) and resuspended in DMEM F-12 (Thermo Fisher Scientific, USA) to a concentration of 1×10^9 cfu/ml. 150 μl of this bacterial suspension were incubated with 1.5 μg recombinant CLEC3A for 5 min, 30 min, 4 h, 8 h, and 24 h at 37°C. The mixtures were centrifuged for 5 min at 4500 g, and supernatants were analyzed by immunoblot.

SDS-PAGE and immunoblot

SDS-PAGE was performed using 4–12% Bis-Tris polyacrylamide gels under reducing conditions. Immunoblotting was performed by transferring the proteins to a polyvinylidene fluoride membrane (Thermo Fisher Scientific, USA). Immunoblotting was performed as previously described[10] by incubating the membrane with affinity-purified polyclonal rabbit antibodies against human CLEC3A. The antibody's specificity for CLEC3A proteins was shown previously.[10] The binding capacity for the CLEC3A peptides Ex1 and Ex12 was validated by immunoblot. The antibody showed reactivity against Ex12, but not against Ex1.

Preparation and coating of titanium substrates

Titanium substrates were prepared and coated with peptides as previously described.[23,24] Briefly, the sequence of the titanium binding peptide (TiBP), which is known to bind titanium with high affinity,[25] and a spacer consisting of three glycine residues (GGG) were added N-terminally to the CLEC3A-derived peptides to be able to coat them to the titanium substrates (Table I). Titanium foil, 0.5 mm thick and 99% pure was cut into 1 cm × 1 cm plates which were washed, transferred to wells of a 24-well plate, and sterilized on each side by ultraviolet germicidial irradiation. They were subsequently coated with 20μM solutions of TiBP, Ex1, Ex12, TiBP-Ex1, and TiBP-Ex12 in PBS or PBS alone. A successful coating of various TiBP-chimeric peptides has already been shown.[26]

Bacterial adhesion on titanium substrates

Bacteria carrying green fluorescence protein-expressing vectors with an ampicillin resistance gene were grown to mid-logarithmic phase in tryptic soy broth (Sigma–Aldrich, Germany) containing 100 μg/ml ampicillin. They were washed with PBS by centrifugation at 4500 *g*. Bacteria were incubated and fixed on titanium substrates as previously described.[23,24] The adherent fluorescent bacteria were visualized using a confocal laser scanning microscope (Leica TCS SP8). The displayed images show representative results from at least three independent experiments, performed in duplicate. Quantification of bacterial adhesion originates from three independent experiments performed in duplicate and was performed by counting bacteria on nine different microscopic images per experiment and peptide. The standard deviation was calculated for each displayed value.

MTT cell viability assay

Primary human chondrocytes (PHC) were isolated from cartilage obtained from three patients, who underwent joint replacement surgery due to coxarthrosis (two patients)or gonarthrosis (third patient) using 0.4% Pronase for 90 min (Calbiochem, USA) as previously described.[27] PHCs (1.13×10^5 or 1.5×10^5 cells/well of a 96-well plate) were cultured for 3 days in dulbecco's modified eagle medium (DMEM) F-12 (Thermo Fisher Scientific, USA) containing 10% Nu Serum (Beckton Dickinson, USA) and 50 μg/ml gentamicin (Thermo Fisher Scientific, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously described.[28] Briefly, the cells were incubated for 2 h in a humidified chamber with 100 μL of serum-free medium containing 20, 10, 5, or 2.5 μM peptide solutions, 2% Triton X-100 or serum-free medium alone (control). The results are averages from three independent experiments performed in duplicates, except for those for Triton X-100, which are averages from two independent experiments performed in octuplets. All results are shown as a percentage of the absorbance measured in the control. The standard deviation was calculated for each value.

Statistical analysis

All graphs contain data originating from three to four independent experiments, performed either in duplicate or triplicate as indicated at the corresponding experiment. All values were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism, version 8.0. The *P*-values were determined using Kruskal–Wallis test. Correction for multiple comparisons was performed using Dunn's test.

Results

Peptide design

Analysis of CLEC3A amino acid sequence showed that it possesses segments with characteristics of AMPs. Typical AMPs consist of 10–50 amino acids, display an overall positive charge (+2 to +11) and possess a considerable number of hydrophobic residues. Their secondary structure is α-helical, β-sheet or a random-coil structure.[29] For this study, we designed two potential AMPs

Table I
List of recombinant proteins and synthetic peptides used in this study. Size and net charge at pH 7 was calculated using www.pepcalc.com whereas the alpha helix content was calculated using NPS@: Network Protein Sequence Analysis

Protein/peptide	Aminoacid position	Aminoacid sequence	Size (Da)	Net charge	Alpha helix
Ex23	39–197	Exon 2 and 3 with N-terminal 8His-tag	17,892.08	−1.1	17.61%
Ex3	68–197	Exon 3 with N-terminal 8His-tag	14,681.49	0.9	7.63%
flc	23–197	Full-length protein with C-terminal double Strep-tag	19,890.43	7.1	16.00%
Ex1	23–38	HTSRLKARKHSKRRVR	2016.37	8.2	18.75%
Ex1z		STSQLQAQKSSQQQVQ	1775.87	1	0%
Ex2	39–67	DKDGDLKTQIEKLWTEVNALKEIQALQT	3228.6	−2	60.71%
Ex12	23–69	HTSRLKARKHSKRRVRDKDGDLKTQIEKLWTEVNALKEIQALQTVCL	5542.39	6.1	61.7%
TiBP		RPRENRGRERGL	1495.65	3	0%
TiBP-Ex1		RPRENRGRERGLGGGHTSRLKARKHSKRRVR	3665.15	11.2	29.03%
TiBP-Ex12		RPRENRGRERGLGGGHTSRLKARKHSKRRVRDKDGDLKTQIEKLWTEVNALKEIQALQTVCL	7191.18	9.1	56.45%

derived from the CLEC3A sequence (Fig. 1, Table I). Namely, CLEC3A-Ex1 (Ex1), containing the positively charged N-terminal sequence and CLEC3A-Ex12 (Ex12), containing the positively charged N-terminal sequence and the alpha-helical OD. By analysis of the CLEC3A amino acid sequence using the ProP 1.0 Server, the predicted furin cleavage site is after position 38, which is the end of the Ex1 sequence.[30] Moreover, MMP-7 cleavage sites have been shown to be toward the end of the Ex12 sequence,[12] indicating that these peptides are likely to be present in cartilage. Since positive charge has been shown to be crucial for antimicrobial activity of most AMPs,[31] we designed CLEC3A-Ex2 (Ex2), which contains the alpha-helical OD without the positively charged sequence as a negative control. In addition, Ex1z was designed as a direct negative control for Ex1, where all positively charged amino acids were substituted by uncharged ones.

Antimicrobial activity

Viable count assay was performed to investigate the antimicrobial activity of CLEC3A-derived peptides [Fig. 2(a)]. Therefore, bacteria were incubated with the peptides and subsequently plated on agar plates. The CLEC3A-derived potential AMPs Ex1 and Ex12 caused a marked inhibition of bacterial growth of *E.coli*, *P.aeruginosa* and *S.aureus*, in concentrations from 2.5 μM to 10 μM, comparable to the reference AMP LL-37, a well investigated AMP with potent antimicrobial, chemotactic and immune modulatory activity.[32,33] The control peptides Ex2 and Ex1z, which do not contain the positively charged sequence, did not exhibit antimicrobial activity, confirming that a positive charge is needed for this activity. For comparison, only the results with peptides in 10 μM concentrations are shown.

Viable count assay was also used to investigate the antimicrobial activity of CLEC3A domains [Fig. 2(a)]. Therefore, recombinantly expressed C-terminal protein domains Ex23 and Ex3, which encompass the OD and the carbohydrate-recognition domain (CRD), or the CRD alone, respectively, were tested against *E.coli* in same molar concentrations as the peptides but did not have an antimicrobial effect. Due to low protein yields, experiments with full-length CLEC3A were conducted using concentrations of up to 2.5 μM. Despite the presence of the positively charged domain, these concentrations of full-length CLEC3A did not lead to an antimicrobial effect against *E.coli*, whereas the CLEC3A-derived peptides did [shown for Ex12, Fig. 2(a)]. Since Ex23, Ex3 and full-length CLEC3A did not exhibit an antimicrobial activity against *E.coli*, they were not tested against *P.aeruginosa* and *S.aureus*.

Membrane permeabilization

Membrane permeabilization is a common microbicidal mechanism of AMPs. To investigate whether CLEC3A peptides also employ this bacterial killing mechanism, fluorescence microscopy was performed [Fig. 2(b)]. Bacteria were first incubated with peptides for membrane permeabilization to occur. After incubation, the addition of FITC to bacteria caused an uptake of the fluorescence dye by the bacteria that was assessed by fluorescence microscopy. The reference AMP LL-37 and the CLEC3A-derived AMPs Ex1 and Ex12 permeabilized the membranes of Gram-negative *E.coli* and *P.aeruginosa.* Ex12 also permeabilized the membranes of Gram-positive *S.aureus*. As expected, after incubation with water (control), or the control peptides Ex1z, or Ex2, no membrane permeabilization occurred. Light microscopy was performed on all samples to confirm the presence of bacteria (results not shown).

Membrane permeabilization was also visualized using scanning electron microscopy [Fig. 2(c)]. Such visualization provides reliable analysis of bacterial cell membrane permeabilization.[14] After

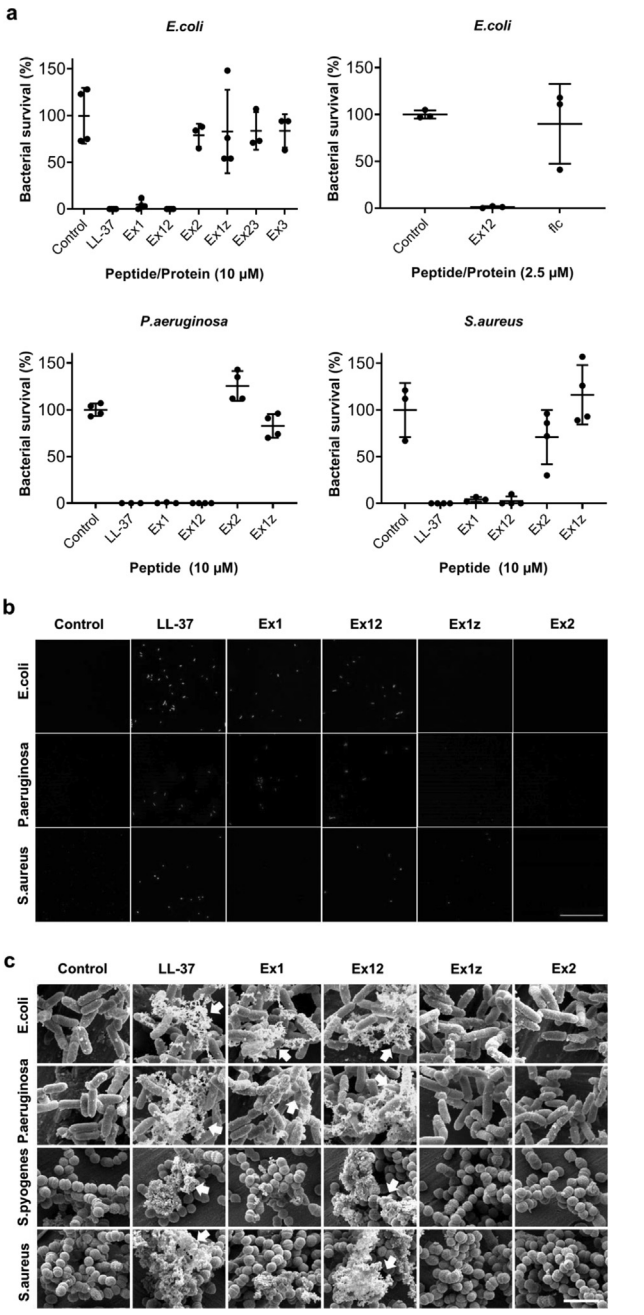

Fig. 2. CLEC3A-derived peptides inhibit bacterial growth and cause permeabilization of bacterial membranes (a) 10 or 2.5 μM of peptide/protein were incubated with *E.coli, P.aeruginosa or S.aureus*. Individual results of colonies grown after incubation with water (control) from 3 to 4 independent experiments are shown. The standard deviation was calculated for each value and is represented by the error bars. (b) Bacteria were incubated with water (control) or 20 μM of synthetic peptides, and subsequently with fluorescein isothiocyanate (FITC). Bacteria with permeabilized cell membranes take up FITC and emit fluorescence (seen in green). Bacteria with intact cell membranes do not take up the dye. The scale bar length is 40 μm. (c) Bacteria were incubated with 20 μM of synthetic peptides or water (control) and visualized using scanning electron microscopy. Membrane perturbation leads to release of cell content which can be seen as a white mass around the bacteria indicated by the white arrows. The scale bar length is 2 μm. The displayed images in (b) and (c) show representative results from at least three independent experiments.

incubation with the CLEC3A-derived AMPs Ex1 and Ex12, Gram-negative *E.coli* and *P.aeruginosa* ruptured, thereby releasing cell content which can be seen as a white mass indicated by the white arrows [Fig. 2(c)]. Ex12 also caused the rupture of Gram-positive

S.pyogenes and *S.aureus*, an effect comparable to the reference AMP LL-37. Incubation with water (control) or the control peptides Ex1z and Ex2 led to no changes in bacterial morphology.

Lipopolysaccharide (LPS) binding

A common mechanism of AMPs is the binding of LPS on the membrane of Gram-negative bacteria. LPS-binding of CLEC3A-derived AMPs was first investigated using transmission electron microscopy. Ex1 and Ex12 were labeled with gold particles, incubated with LPS, and visualized using a transmission electron microscope [Fig. 3(a)]. After incubation with the gold-labeled reference AMP LL-37 and the CLEC3A-derived peptides Ex1 and Ex12, the gold particles could be observed on the LPS surface, demonstrating LPS-binding of these peptides. When incubated with water (control), or the control peptides Ex1z, or Ex2, no gold particles could be seen.

Additionally, LPS-binding was investigated on *E.coli* membranes. Antibodies against *E.coli* LPS conjugated to gold particles 10 nm in diameter and synthetic peptides conjugated to gold particles 5 nm in diameter were visualized using a transmission electron microscope [Fig. 3(b)]. Upon incubation with gold-labeled reference AMP LL-37, or CLEC3A-derived AMPs Ex1, or Ex12, the small gold particles (marked by white arrows) were seen co-localized with the large gold particles (marked by black arrows) (antibodies against LPS), showing an LPS binding of these peptides. Furthermore,

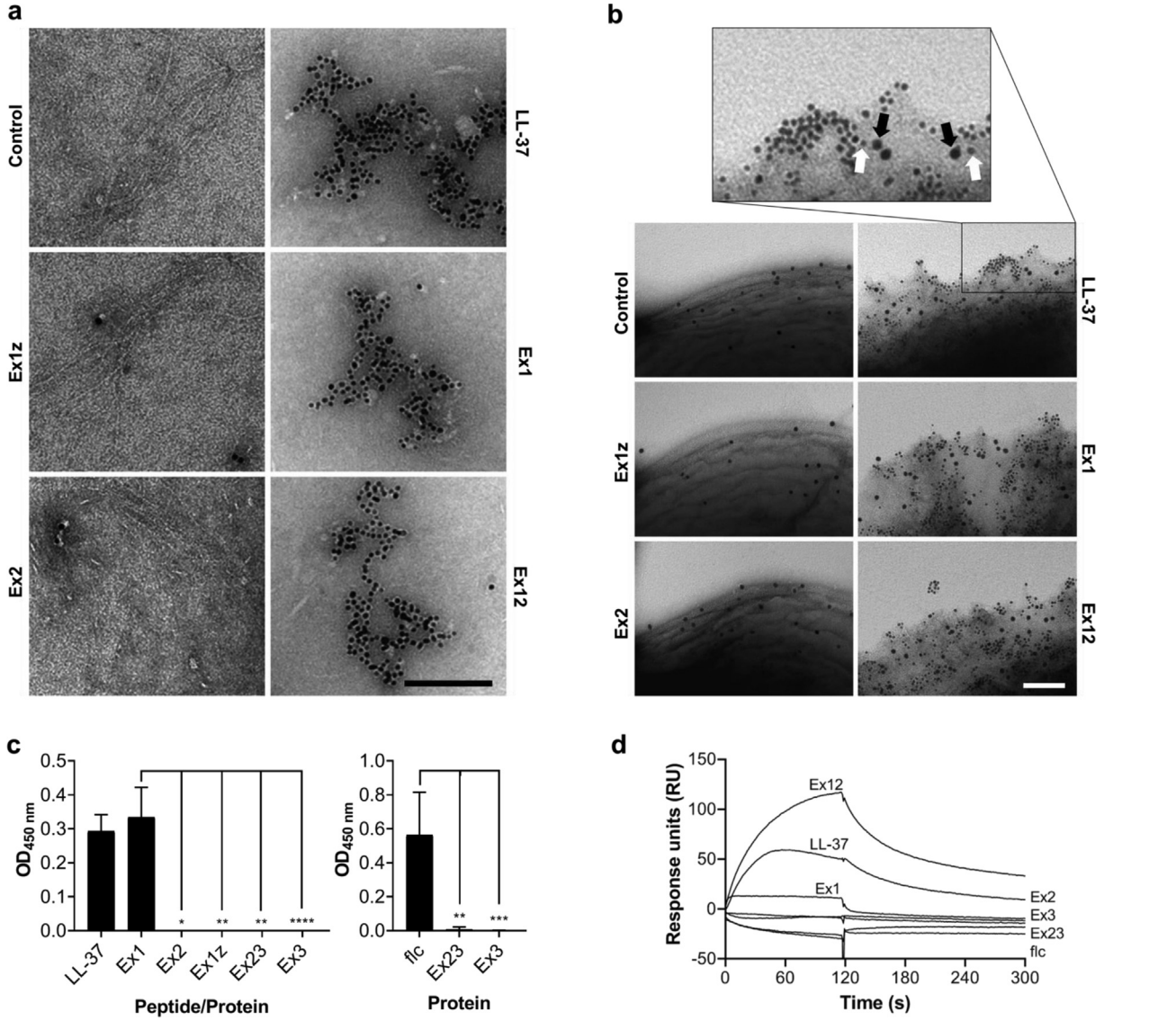

Fig. 3. Full-length CLEC3A (flc) and CLEC3A-derived peptides bind LPS and lipoteichoic acid (LTA) on bacterial membranes and cause membrane deformation. (a) LPS was incubated with gold-labeled synthetic peptides or water (control) and subsequently visualized using an electron microscope. The presence of clustered black dots represents the gold-labeled peptides having bound to LPS. The scale bar length is 100 nm. (b) Antibodies against *E.coli* LPS were labeled with 10 nm gold particles (seen as larger dots marked by black arrows) whereas the synthetic peptides were labeled with 5 nm gold particles (seen as smaller dots marked by white arrows). *E.coli* membranes were visualized using an electron microscope after incubation with gold-labeled antibodies against LPS and gold-labeled synthetic peptides or water (control). The scale bar length is 100 nm. The displayed images in (a) and (b) show representative results from at least three independent experiments. (c) Synthetic peptides or recombinant proteins were coated to wells of 96-well plates, incubated with biotin-labeled LPS, and detection performed using streptavidin-peroxidase (left). Ex12 was left out of this experiment due to an unspecific binding to streptavidin. LPS was coated to wells of 96-well plates, incubated with the recombinant proteins, which were detected using an antibody against human CLEC3A (right). The results are shown as averages from at least three independent experiments performed in triplicate. Standard deviation was calculated for each value and is represented by the error bars. $^{*}P < 0.05$, $^{**} < 0.01$, $^{****}P < 0.0001$. (d) For surface plasmon resonance, *S.aureus* LTA was immobilized on a sensor chip and 300 nM of the indicated peptides were flown over the chip upon injection. Experiments were performed in duplicate.

incubation with the LPS-binding peptides LL-37, Ex1, or Ex12 caused a disruption and slight fragmentation of the bacterial cell membrane. When incubated with water (control), or gold-labeled control peptides Ex1z, or Ex2, only the 10 nm-gold particle-labeled antibodies against LPS could be observed on the bacterial membrane.

To determine whether full-length CLEC3A and its domains bind LPS, an LPS-binding assay was performed. The CLEC3A-derived AMP Ex1 bound LPS, to an extent comparable to that of the reference AMP LL-37 [Fig. 3(c), left panel]. Ex12 showed unspecific binding to streptavidin used for detection in the LPS-binding assay and is therefore not shown here. The control peptides Ex2, Ex1z and the recombinant CLEC3A domains Ex23 and Ex3 exhibited no LPS binding. Since full-length CLEC3A contains a C-terminal Strep-tag, its binding to LPS was tested as described in the Methods section. Full-length CLEC3A bound LPS whereas Ex23 and Ex3 did not [Fig. 3(c), right panel].

Lipoteichoic acid (LTA) binding

A common AMP target on the membranes of Gram-positive bacteria is LTA.[34,35] LTA binding was investigated by surface plasmon resonance [Fig. 3(d)]. Upon passing of Ex12 over the LTA-immobilized chip, a strong binding could be detected. LL-37 was used as a reference. Ex1 showed weak to no binding, whereas for Ex2, Ex3, Ex23 and full-length CLEC3A no binding was detected.

Physiological relevance

To investigate whether CLEC3A-derived peptides are present in cartilage, human hip cartilage extracts were analyzed by immunoblot using antibodies against human CLEC3A. CLEC3A–derived peptides similar in size to the CLEC3A-derived antimicrobial peptide Ex12 were detected [Fig. 4(a)]. Moreover, upon incubation of recombinant CLEC3A with *P.aeruginosa*, a time-dependent cleavage of the protein could be observed [Fig. 4(b)]. Cartilage-derived CLEC3A was also proteolytically cleaved after incubation with *P.aeruginosa* [Fig. 4(c)].

Translational application

The increased number of performed arthroplasties is accompanied by increasing numbers of septic arthritis cases.[36] We therefore assessed the application of CLEC3A-derived AMPs to prevent bacterial adhesion to joint prostheses. We coated titanium substrates, a common joint prostheses material, with CLEC3A-derived peptides. To facilitate binding of CLEC3A-derived AMPs to titanium substrates, we designed two chimeric CLEC3A-derived peptides, consisting of a titanium binding peptide (TiBP), which has been shown to bind titanium with high affinity[25] and the sequence of CLEC3A-derived AMPs Ex1 (TiBP-Ex1) or Ex12 (TiBP-Ex12) (Table I). Coating titanium substrates with CLEC3A-derived chimeric peptides visibly reduced the number of adhering bacteria [Fig. 5(a)]. Bacterial adhesion was quantified for uncoated titanium substrates (PBS) and those coated with TiBP, TiBP-Ex1 or TiBP-HT-Ex12. These results show that coating titanium substrates with TiBP-Ex12 leads to the most prominent decrease in bacterial adhesion [Fig. 5(b)].

To test whether CLEC3A peptides display a direct cytotoxic effect in cartilage, an MTT cell viability assay was performed by incubating PHC with the peptides (Fig. 6). These experiments showed that microbicidal concentrations of CLEC3A peptides do not display a direct cytotoxic against PHC.

Discussion

Here, we demonstrate that peptides of the cartilage-specific CLEC3A kill septic arthritis-causing bacteria *S.aureus, E.coli, and P.aeruginosa*, showing that CLEC3A-derived peptides are cartilage-specific AMPs. They do so by binding to lipopolysaccharides on membranes of Gram-negative bacteria or LTA on membranes of Gram-positive bacteria which leads to bacterial membrane permeabilization. This mechanism is analogous to that of major groups of AMPs.[7,34,35,38]

Release of CLEC3A-derived AMPs to fight infection is likely to occur physiologically. CLEC3A is known to be highly susceptible to cleavage by cartilage proteases.[12] CLEC3A's sequence contains cleavage sites for furin and MMP-7. A furin cleavage would result in

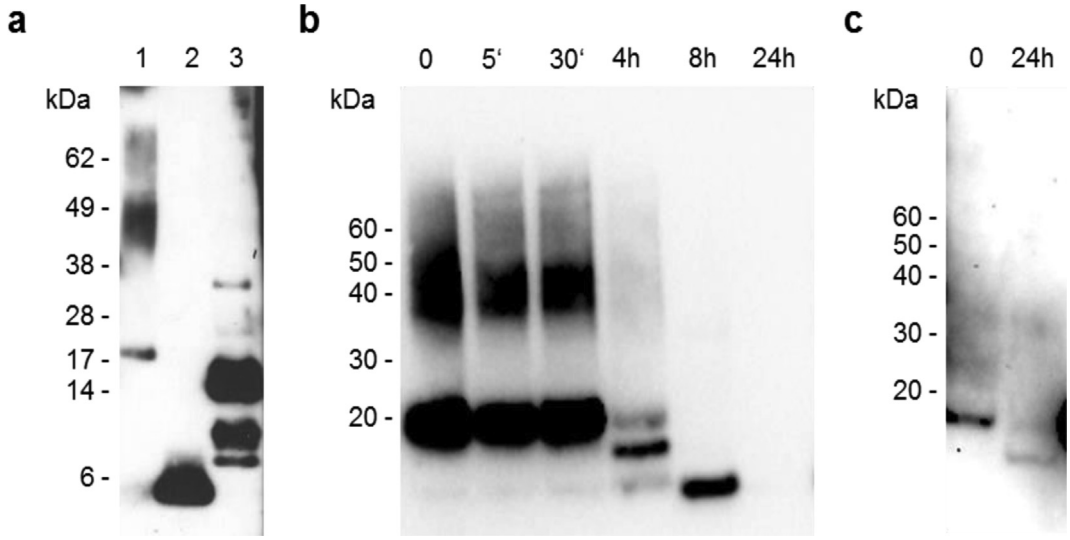

Fig. 4. AMP-like CLEC3A peptides present in human articular cartilage and after bacterial cleavage of CLEC3A. CLEC3A-derived peptides were detected in cartilage and after incubation of recombinant CLEC3A with *P.aeruginosa* culture supernatants using immunoblot. Human articular cartilage originates from one patient sample. (a) 15 ng of recombinant full-length CLEC3A (lane 1), 2 μg of Ex12 (lane 2) and human hip cartilage extracts (lane 3). (b) *P.aeruginosa* culture supernatants incubated with 230 ng of CLEC3A for the indicated time points. (c) Human hip cartilage extracts before and after 24-h incubation with *P.aeruginosa*.

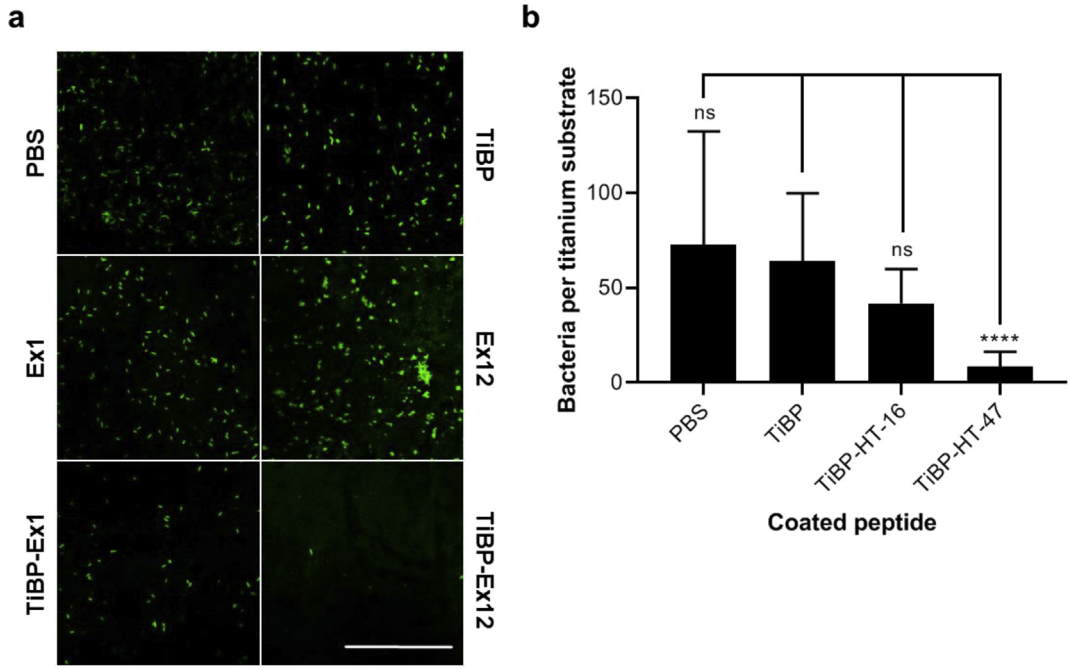

Fig. 5. Coating titanium substrates with the CLEC3A-derived chimeric antimicrobial peptides (AMPs) reduces bacterial adhesion. (a) Titanium substrates coated with peptides or uncoated (PBS) were incubated with *E.coli* containing a green fluorescent protein (GFP)-expressing vector. The adherent bacteria emit a green fluorescent signal when visualized using a fluorescent microscope. The scale bar length is 50 μm. (b) Results are shown as bacterial count averages from three independent experiments, performed in duplicate. Bacterial counts originate from nine microscopic images per experiment and peptide. The standard deviation was calculated for each value and is represented by the error bars. ****$P < 0.0001$, ns − non significant.

a peptide containing the amino acid sequence of the CLEC3A AMP Ex1, whereas MMP-7 cleavage would result in a peptide containing the amino acid sequence of Ex12. Our results show that CLEC3A-derived peptides, similar to the designed AMPs, can indeed be detected in cartilage. The peptides detected in cartilage, however, are not identical to the designed CLEC3A-derived AMPs. One reason for this could be CLEC3A's susceptibility to cleavage by other cartilage proteases, which could result in different peptides. Furthermore, it is likely that the antibodies, which were raised against the full-length protein, do not detect all physiologically present CLEC3A fragments. Nonetheless, AMP-like CLEC3A-derived peptides are present in cartilage, suggesting that CLEC3A plays a role in the cartilage-specific innate immune response.

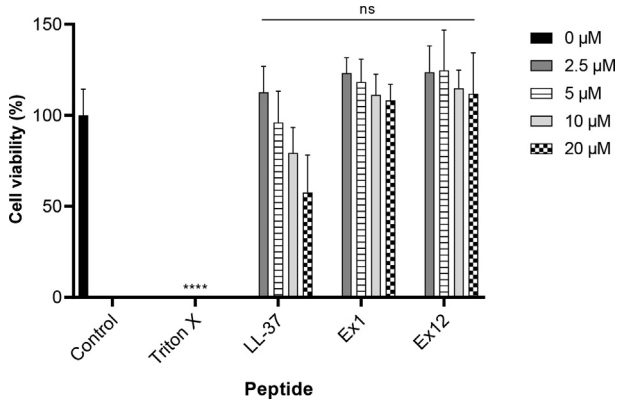

Fig. 6. Viability of primary human chondrocytes is not affected by bactericidal concentrations of CLEC3A peptides. Cells isolated from articular cartilage originating from three patients were incubated with the indicated peptide concentrations or with Triton X-100, which served as a control for a total loss of cell viability. Results are shown as percentages of absorption values of cells after incubation without peptides (control) from three independent experiments performed in triplicate. The standard deviation was calculated for each value and is represented by the error bars. ****$P < 0.0001$, ns − non significant.

Recent work has shown that CLEC3A activates plasminogen.[10] The expression of both CLEC3A and plasminogen has been shown to be induced by proinflammatory cytokines.[39,40] It is therefore tempting to speculate that higher CLEC3A concentrations during inflammation could activate plasminogen, which could in turn lead to fragmentation of CLEC3A into AMPs. This would indicate a potential role of plasminogen in cartilage immune response.

Bacteria also cause cartilage degradation by secretion of proteolytic enzymes.[36] Our results confirm these findings and show that *P.aeruginosa* proteases cleave recombinant and cartilage-derived CLEC3A. Bacterial proteases are most likely a bacterial protection mechanism against the immune system, but could also, at least transiently, lead to the formation of AMPs from their precursors.

The increasing bacterial resistance toward antibiotics is drastically limiting the number of available therapies. Intensive research is therefore aiming at identifying novel effective therapeutics. AMPs are a promising focus of such research, proving to be efficient even in clinical trials.[29] One specific clinical application of CLEC3A-derived AMPs could be in the treatment of septic arthritis, which currently requires draining of purulent fluid from the infected joint by needle aspiration or surgical procedures and subsequent antibiotic treatment.[36] CLEC3A-derived peptides could be used either in combination with antibiotics or alone. A major drawback of antibiotics and chemically synthesized non-endogenous peptides is their immunogenicity.[41] Due to the presence of CLEC3A-derived peptides in cartilage under physiological conditions, these are less likely to be immunogenic. Moreover, high antibiotic concentrations are often toxic.[42] In contrast, microbicidal concentrations of CLEC3A-derived peptides show no direct cytotoxicity against PHC, so these cartilage-specific AMPs could be used as a non-immunogenic and non-toxic alternative in the treatment of infection, especially of septic arthritis.

A major, increasingly important cause of septic arthritis is arthroplasty. During arthroplasty, bacteria can adhere to implant surfaces and therefore be introduced into the patient's joint.[36] Moreover, the surfaces of biomaterials used as implants (such as titanium) are susceptible to biofilm formation. Adhesion of bacteria contributes to biofilm formation, which is a structured aggregation of bacteria encased in a self-produced matrix. Bacterial cells inside such biofilms grow slowly or do not grow at all, which is one of the main reasons why antibiotics show poor activity against these infections.[43] Consequently, avoiding biofilm formation would be an effective way to prevent infections. Our experiments show that coating titanium substrates with CLEC3A-derived peptides prevents bacterial adhesion to the substrates. Since the peptides exhibit an antimicrobial effect, bacterial killing is the most likely mechanism of this finding. Coating joint prostheses with CLEC3A-derived AMPs could therefore be a prophylactic application preventing bacterial adhesion and thereby biofilm formation.

The main limitation of our study is the modest number of patient samples used. Further investigation is therefore required before our findings can be generalized to a wider population.

Taken together, we identify AMPs derived from the cartilage-specific protein CLEC3A, describe the mechanism of their antimicrobial activity and suggest novel approaches in the prevention and treatment of septic arthritis using potent, non-toxic cartilage-specific AMPs.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Klatt had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Elezagic, Klatt.

Acquisition of data: Elezagic, Mörgelin, Hermes, Hamprecht, Sengle, Lau, Höllriegl.

Analysis and interpretation of data: Elezagic, Mörgelin, Hamprecht, Sengle, Wagener, Paulsson, Streichert, Klatt.

Conflict of interest

There are no conflicts of interest.

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