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ORIGINAL ARTICLE

The antimicrobial peptide HBD-2 and the Toll-like receptors-2 and -4 are induced in synovial membranes in case of septic arthritis

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Abstract Septic arthritis is frequently observed especially in immune-compromised or chronically diseased patients and leads to functional impairment due to tissue destruction. Recently, production of antimicrobial peptides (AMP) was observed in articular cartilage after exposure to bacteria. This report examines the role of synoviocyte-derived AMPs in innate defense mechanisms of articular joints. Samples of healthy, low-grade synovialitis and septic synovial membranes were assessed for the expression of human β-defensin-2 (HBD-2) and Toll-like receptor-2 and -4 (TLR) by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). A stable synoviocyte line (K4IM) was used for in vitro experiments and assayed for endogenous HBD-2 and TLR production after exposure to inflammatory cytokines or bacterial supernatants by reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, Western blot, ELISA, and dual luciferase assay. Healthy human synovial membranes and cultured synoviocytes are able to produce HBD-2 and TLR-1–5 at basal expression levels. Samples of bacteria-colonized synovial membranes produce higher levels of HBD-2 when compared with samples of healthy tissues. K4IM synoviocytes exposed to *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or proinflammatory cytokines demonstrated a clear HBD-2 transcription and protein induction. TLR-2 and -4 are known to have a critical role in the recognition of gram-positive and gram-negative bacteria in epithelia and are induced in mesenchymal synoviocytes after bacterial exposure on transcription and on protein level. This report demonstrates an unappreciated role of synovial membranes: samples of septic synovial membranes and cultured synoviocytes exposed to bacteria produce increased amounts of the AMP HBD-2 and the bacteria recognition receptors TLR-2 and -4. The induction of anti-inflammatory pathways in infected synoviocytes suggests involvement in intra-articular defense mechanisms.

Keywords Bacterial infection · Antimicrobial peptide · Human β-defensin-2 · Toll-like receptor

The experiments comply with the current laws of Germany.

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Introduction

Septic arthritis frequently results from hematogenous spread of bacteria or traumatic or surgical bacterial contamination. The most common isolated bacteria in this case are *Staphylococcus aureus* [1, 2]. Once the bacteria invade the synovial membrane, bacterial toxins stimulate the release of multiple cytokines such as tumor necrosis factor (TNF-α) or interleukin-1 (IL-1). These cytokines, in

turn, induce the production of proteolytic enzymes in synovial membrane and support the destruction of articular cartilage [3–5].

In general, the synovium consists of a thin lining layer of macrophages and fibroblasts [6, 7]. In healthy organisms, the predominant cell type is of mesenchymal origin and demonstrates fibroblast-like features [8, 9]. In pyogenic arthritis, the synovial lining thickens, and the sublining tissue becomes infiltrated with T cells, B cells, and macrophages.

The host response to bacterial infection is dependent on both innate (non-antibody-mediated) and adaptive (antibody-mediated) immune systems. The adaptive immune system is primarily cellular in composition and relies on the actions of B and T cells. The innate immune response is more immediate and depends on the activity of phagocytic cells and the expression of a number of antimicrobial proteins and peptides (AMP) [10, 11]. Defensins are an important subfamily of AMP and are able to kill microbes by destructing their cell membranes. To date, six human β-defensin (HBDs), HBD-1 through -6, have been identified in human tissues [10, 11]. The HBDs differ in tissue distribution and expression profile after stimulation with proinflammatory cytokines or bacteria [12–16]. The HBD-2 was first isolated from human skin and displays potent antimicrobial activity in the gram-negative and -positive range as well [12]. The importance of AMP in host defense becomes evident in mice overexpressing the human alpha-defensin-5 gene and the subsequent resistance to oral application of *Salmonella typhimurium* [17]. Other studies revealed that mice with disrupted AMP genes are prone to infection in the affected organs [18].

Toll-like receptors (TLR) are a family of transmembrane receptors [19–21]. They were regarded as key regulators of both innate and adaptive immune response and recognize pathogen-associated molecular patterns (PAMPs) from gram-positive and -negative bacteria [22]. The interaction of TLRs with PAMPs results in the nuclear translocation of the transcription factor nuclear factor kappa B (NF-κB) and a subsequent increase of AMP production [9, 10, 23]. Studies on epithelia demonstrate that TLR-4 and its accessory molecule lymphocyte antigen 96 (MD-2) are required for the recognition of lipopolysaccharide found in the membranes of gram-negative bacteria [24]. By contrast, TLR-2 is required for the recognition of bacterial lipopeptide, and in combination with TLR-6, for the detection of peptidoglycan and lipoteichoic acid, which are components of gram-positive bacteria [23]. Up to now, reports are missing concerning the expression and regulation of TLRs in mesenchymal synoviocytes in case of bacterial exposure.

Recent studies of our group have demonstrated that synovial membranes have the ability to produce HBDs in case of inflammatory or bacterial joint disease [25, 26]. Moreover, expression pattern of these peptide antibiotics changed, dependent on the kind of joint disease, thus, suggesting a regulative AMP production after being challenged by inflammatory mediators [26].

The aim of the current study was to determine inflammatory production and regulation of HBD-2 in human synovial membranes and to evaluate the findings in an in vitro model with immortalized synoviocytes called K4IM and primary synoviocytes.

Material and methods

Tissues

Healthy synovial membranes (n=6) were collected from knee joints without signs of degeneration. The articular joints were dissected from body donors, donated to the Institute of Anatomy. Infected synovial membranes (high-grade synovialitis; n=6) and low-grade synovialitis synovial membrane (n=6) were collected from patients who underwent revision surgery due to bacterial infection at the Department of Trauma, University of Kiel. All samples from patients suffering from septic arthritis showed positive microbiological cultures for gram-positive bacteria such as *S. aureus* (n=3) or *S. epidermidis* (n=3). The study was approved by the institutional review board.

Human cell culture

To analyze parameters that lead to the activation of synovial fibroblasts, we cultured a stable human synoviocyte line (K4IM, a generous gift from Christian Kaps, Charite, Berlin, Germany) which is immortalized with the SV40 T antigen [27]. Several studies confirmed that the immortalized K4IM cell line represents a valuable tool to study mechanisms that induce synoviocyte activation [27, 28]. For Western blot analysis, primary synoviocytes were collected from healthy synovial membranes of body donors and prepared for in vitro examinations, as recently described by Ralph et al. [29]. For in vitro experiments, synoviocytes were cultured in monolayers in RPMI-1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, and 50 μg/ml penicillin-streptomycin (Gibco BRL). At 80% confluency, stimulation experiments were performed in serum-free RPMI-1640 medium in humidified 5% CO_2 atmosphere.

Stimulants

Synoviocytes in monolayer culture were exposed to IL-1/-6 (10 ng/ml), TNF-α (10 ng/ml) or supernatants of *Pseudomonas aeruginosa* (PAS) or *S. aureus* (diluted 1:50) for 6 or

24 h. The supernatants were generated from clinical isolates, as recently described by Gläser et al. [30].

RNA preparation and cDNA synthesis

Frozen tissue samples (20 mg) of healthy and infected synovial membranes were crushed in an achate mortar under liquid nitrogen. RNA from tissues was generated by Trizol reagent. Moreover, RNA from cultured synoviocytes was extracted with the RNeasy Total RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Contaminating DNA was destroyed by digestion with RNase-free DNase-I (20 min at 25°C, Boehringer, Mannheim, Germany). After inactivation of DNase (15 min at 65°C), complementary DNA (cDNA) was generated with 1 µl (20 pmol) of oligo (dt) primer (Amersham Pharmacia, Uppsala, Sweden) and 0.8 µl of superscript RNase H-reverse transcriptase (Gibco, Paisley, UK) for 60 min at 37°C.

Reverse transcription polymerase chain reaction

For PCR, 4 µl of cDNA were incubated with 30.5 µl water, 4 µl 25 mM $MgCl_2$, 1 µl dNTP, 5 µl 10 × PCR buffer, and 0.5 µl (2.5 U) platinum *Taq* DNA polymerase (Gibco), and the following pairs of primers: HBD-2-for1 5′-CCAGCCAT CAGCCATGAGGGT-3′, HBD-2-ra 5′-GGAGC CCTTT CTGAATCCGCA-3′, 57°C, 255 bp; TLR-1-for1 5′-CTATA CACCAAGTTGTCAGC-3′, TLR-1-ra 5′-GTC TCCAACT CAGTAAGGTG-3′, 56°C, 210 bp; TLR-2-for1 5′-GCCAA AGTCTTGATTGATTGG-3′, TLR-2-ra 5′-TTG AAGTTCT CCAGCTCCTG-3′, 56°C, 347 bp; TLR-3-for1 5′-GATCT GTCTCATAATGGCTTG-3′, TLR-3-ra 5′-GAC AGATTCCG AATGCTTGTG-3′, 56°C, 300 bp; TLR-4-for1 5′-TGGATACGTTTCCTTATAAG-3′, TLR-4-ra 5′-GAAATGGAGGCACCCCTTC-3′, 56°C, 548 bp; TLR-5-for1 5′-CTAGCTCCTAATCCTGATG-3′, TLR-5-ra 5′-C CATGTGAAGTCTTTGCTGC-3′, 56°C, 400 bp. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific intron-spanning primer pair (forward primer: 5′TGA AGGTCGGAGTCAACGGA TTTGGT-3′; reverse primer: 5′-CATGTGGGCCATGAGGTCCACCAC -3′), which yielded a 983-bp amplified product, served as the internal control for equal amounts of cDNA. Thirty-five cycles were performed with each primer pair. All primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. For the negative control reaction, the cDNA was replaced with water.

Real-time reverse transcription polymerase chain reaction

Real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using a one-step RT-PCR system (Qiagen; QuantiTect SYBR Green RT-PCR). For this purpose, 100 ng of total RNA was added. Real-time RT-PCR was used to monitor gene expression using an i-Cycler (Biorad, München, Germany) according to the standard procedure. PCR was performed, as recently described by our group [31, 32]. I-Cycler Data Analysis software (Biorad, München, Germany) was used for PCR data analysis. The used TaqMan primers and probes had the following identification numbers: GAPDH: Hs99999905_m1, HBD-2: Hs00823638_m1, TLR-2: Hs00610101_m1, and TLR-4: Hs00370853_m1 (Applied Biosystems, Darmstadt, Germany). Relative quantification was performed by normalizing the signals of the different genes against those of GAPDH. The assessed data included three independent experiments with triplicates.

Western blot

For Western blots, samples were reduced in the presence of 10 mM dithiothreitol, proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10% gels), transferred onto nitrocellulose membranes that were blocked and incubated with antibodies according to standard techniques as described [33]. Signals were detected by chemiluminescence reaction (ECL-Pus; Amersham Pharmacia, Uppsala, Sweden).

HBD-2 enzyme-linked immunosorbent assay

For enzyme-linked immunosorbent assay (ELISA), 100 mg fresh weight of healthy and infected synovial membranes was crushed in an achate mortar under liquid nitrogen and homogenized in 150 mM NaCl, 20 mM Tris HCl buffer, pH 7.4, using a polytron homogenizer (Kinematica, Luzern, Switzerland). A soluble fraction was obtained by centrifugation at 48.000×g for 60 min. Subsequently, 50 µl aliquots of this homogenates and aliquots of the collected cell supernatants from the stimulation experiments were examined by sandwich ELISA. Ninety-six well immunoplates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated at 4°C for 24 h with 100 µl (0.5 µg/ml) goat anti-HBD-2 antibody (Acris, Hiddenhausen, Germany; PP1125P2) diluted 1:500 in 0.05 M carbonate buffer, pH 9.6. Subsequently, wells were blocked with 200 µl 1% bovine serum albumin in phosphate buffer solution (PBS) for 10 min at room temperature. After three times washing with 200 µl PBS+0.1% Tween 20, 100 µl per well of cell culture supernatants were incubated for 30 min at room temperature. Plates were washed thrice with PBS+0.1% Tween 20, and wells were incubated for 30 min at room temperature with 50 µl of biotinylated goat anti-HBD-2 antibody (Acris, Hiddenhausen, Germany, PP1125B1) diluted 1:2.500 to 0.2 µg/ml in PBS+0.1% Tween 20. Plates were washed again three times with PBS+0.1%

Tween and filled with 50 µl/well of streptavidin-POD (Roche Diagnostics, Mannheim, Germany; 1:10.000 in PBS+0.1% Tween 20). The plates were then incubated for 30 min at room temperature, washed three times as described above, and incubated with 2.2′-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (Roche Diagnostics) as the development agent for 15–45 min at room temperature in the dark. Absorbance was measured at 405 nm with a multichannel photometer (Sunrise; Tecan, Crailsheim, Germany). Human recombinant HBD-2 (PeproTec, Rocky Hill, CT, USA) served as the standard with the following concentrations: 0, 0.16, 0.32, 0.64, 1.25, 2.5, and 5 ng/ml.

Dual luciferase assay

The luciferase assay was carried out as described in Harder et al. [34].

Statistical analysis

Differences between the groups were evaluated using the *t* test. Group differences were considered significant if $P< 0.05$. All statistical analyses were carried out using the JMP statistics package (SAS Institute, Cary, NC, USA).

Results

Human β-defensin-2 is induced in septic synovial membrane

To evaluate HBD-2 expression in low-grade synovialitis and in healthy and inflamed human synovial membranes (high-grade synovialitis), RT, PCR, immunohistochemistry, and ELISA experiments were performed.

RT-PCR revealed HBD-2 transcripts in all examined tissue samples of bacteria-infected synovial membranes (Fig. 1a). Immunohistochemistry was used to analyze expression pattern of HBD-2 in healthy and infected tissues. Neglectable immunostaining was demonstrated in tissue samples of low-grade synovialitis or healthy synovial membranes (Fig. 1b), but bacterial colonization results in a significant upregulation of HBD-2. The observed immunostaining was primarily visible in the extracellular matrix of fibroblasts, as detected by their characteristic shape (Fig. 1b).

ELISA experiments were performed to analyze quantitative amounts of HBD-2 protein in the collected tissue samples of healthy, low-grade synovialitis and high-grade synovialitis (bacteria-infected synovial membranes). In case of gram-positive infections, HBD-2 expression levels clearly raised to 1.5 ng/100 mg fresh tissue, thus demonstrating a microbial influence in the regulation of synovial membrane-derived AMP (Fig. 1c). The amount of HBD-2 in healthy and in low-grade synovialitis was similar (Fig. 1c).

IL-1, IL-6, TNF-α, *P. aeruginosa*, and *S. aureus* stimulate HBD-2 expression in cultured synoviocytes

To assess inducers of HBD-2 in synovial membranes, K4IM synoviocytes were taken into cell culture and stimulated with different proinflammatory cytokines and supernatants of a clinical isolate of *S. aureus* or *P. aeruginosa*. After 6 or 24 h of stimulation, RNA or cell supernatants were collected and assayed by RT-PCR, real-time RT-PCR, or ELISA experiments. Similar to recent results in chondrocytes, cultured K4IM synoviocytes strongly induce HBD-2 transcripts after exposure to IL-1/-6 or TNF-α (Fig. 2a, b). Among these inflammatory cytokines, TNF-α has the greatest impact on HBD-2 gene expression in K4IM cells (Fig. 2b). Protein analysis with a HBD-2 sandwich ELISA revealed gram-positive bacteria of *S. aureus* and gram-negative *P. aeruginosa* as additional stimulators, because 24 h after exposure amounts of secreted HBD-2 protein raised up to 160 ng/300.000 cells. Interestingly, induction of HBD-2 was not dependent on the specification of the bacteria and did not exceed amounts of cytokine-exposed synoviocytes (Fig. 2c).

The Toll-like receptors 1–5 are expressed in healthy synovial membranes

To investigate the expression of TLRs, which are known to be involved in the regulation of AMPs in epithelia, RT-PCR, immunohistochemistry, and Western blot investigations were performed. RT-PCR revealed transcripts of TLR-1–5 in homogenates of healthy synovial membranes (Fig. 3), indicating a role in the regulation of AMP in mesenchymal synovial membranes. Because of their key role in bacteria-mediated anti-inflammatory pathways in epithelia, production of TLR-2 and -4 was additionally demonstrated by immunohistochemistry in samples of septic synovial membranes (Fig. 1b). Regulation of TLR-4 was examined in primary synoviocytes after exposure to *P. aeruginosa.* Western blot analysis revealed induction of gram-negative specialized TLR-4 after 24 h of bacterial stimulation.

Bacterial induction of TLR-2 and -4 in cultured synoviocytes

To test the inducibility of TLR-2 and -4 in cultured synoviocytes, K4IM cells were challenged by TNF-α or supernatants of *P. aeruginosa* and *S. aureus.* After 6 h of co-culturing, RT-PCR and real-time RT-PCR examinations were performed. Addition of the proinflammatory cytokine

TNF-α (10 ng/ml) resulted in an increased transcription of TLR-4 (Fig. 4a). Real-time RT-PCR demonstrated a clear induction of TLR-2 and -4-messenger RNA after 6 h of exposure to gram-negative *P. aeruginosa* and gram-positive *S. aureus* (Fig. 4b). Interestingly, bacterial specification did not significantly influence TLR-2 gene expression, but quantitative TLR-4-RT-PCR revealed more transcripts in case of gram-positive bacterial stimulation with *S. aureus* (Fig. 4b).

Proinflammatory cytokines and supernatant of *P. aeruginosa* and *S. aureus* increase promoter activity of HBD-2

In order to verify the ELISA and real-time RT-PCR data, we performed dual luciferase assays. IL-1 leads to an 11-fold, TNF-α to an eightfold, and IL-6 to a twofold increase of the HBD-2 promoter activity (Fig. 5a). *P. aeruginosa* leads to a 1.2-fold and *S. aureus* to a 2.2-fold increase of the HBD-2 promoter activity (Fig. 5b). After 12 h, 10 ng/ml IL-1 leads to a maximum of HBD-2 promoter activity (Fig. 5c; control=1). All experiments were performed with $n=8$.

Discussion

Defensins are an essential part of the host innate immune system responsible for the first line of defense against pathogenic microorganisms [10, 11]. In case of bacterial arthritis, many cell types neighboring to synoviocytes (for instance chondrocytes, osteocytes, and osteoblasts) were

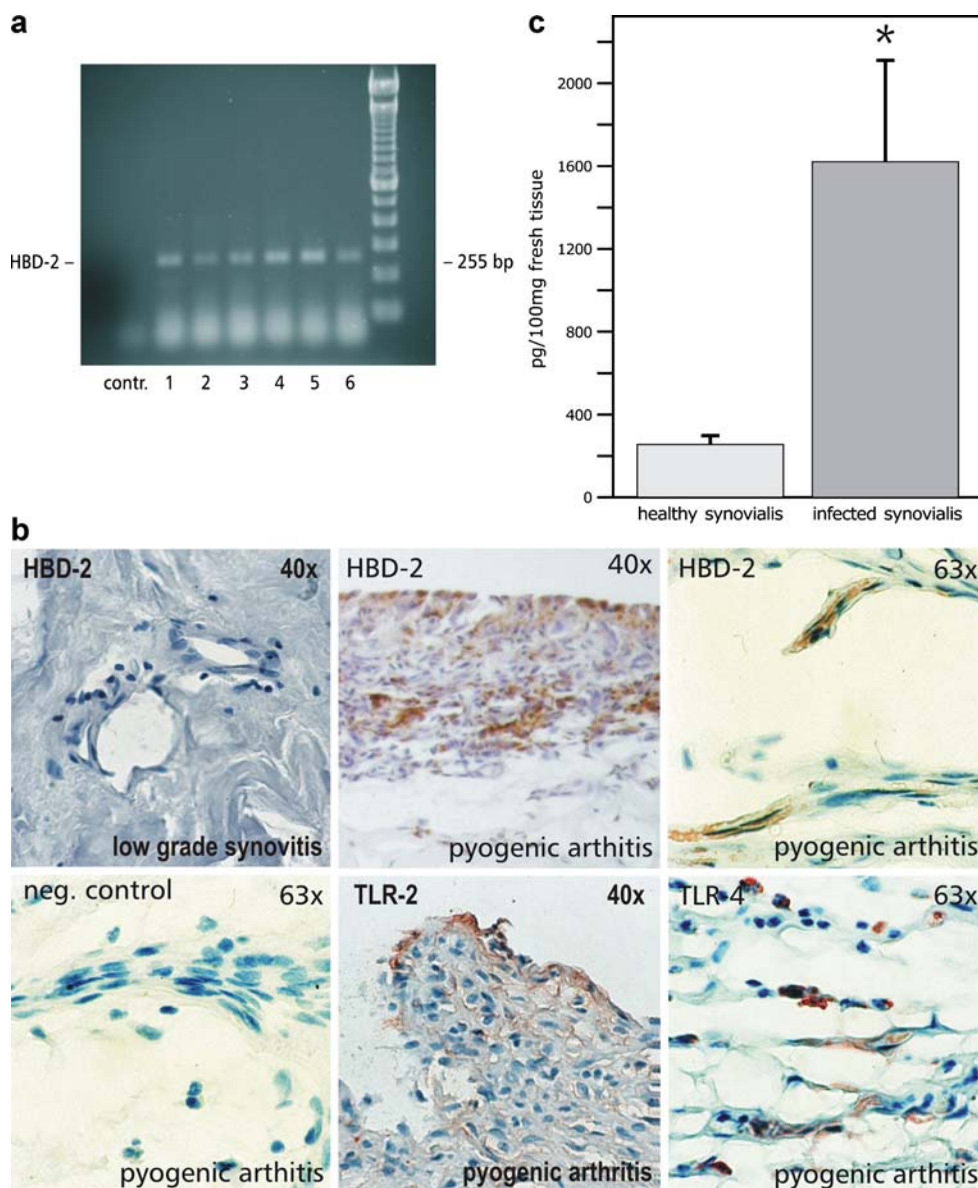

Fig. 1 a–c Human β-defensin-2 is expressed and induced in bacteria-infected synovial membranes. To assess production of HBD-2 in synovial membranes after bacterial infection, RT-PCR, immunohistochemistry, and ELISA experiments were performed. RT-PCR revealed HBD-2 transcripts in all examined bacteria-colonized tissue samples (**a**). Immunohistochemical examinations confirmed increased staining activity in samples of infected synovial membranes. Staining was primarily found in the extracellular matrix of fibroblasts, as detected by their characteristic cigar-shape (**b**). ELISA experiments approved increased expression of the endogenous antibiotic. In case of bacterial colonization with staphylococci, HBD-2 amounts peaked to 1.5 ng/100 mg fresh tissue (**c**). Values are the mean ± standard deviation, $^{*}P<0.05$ versus controls

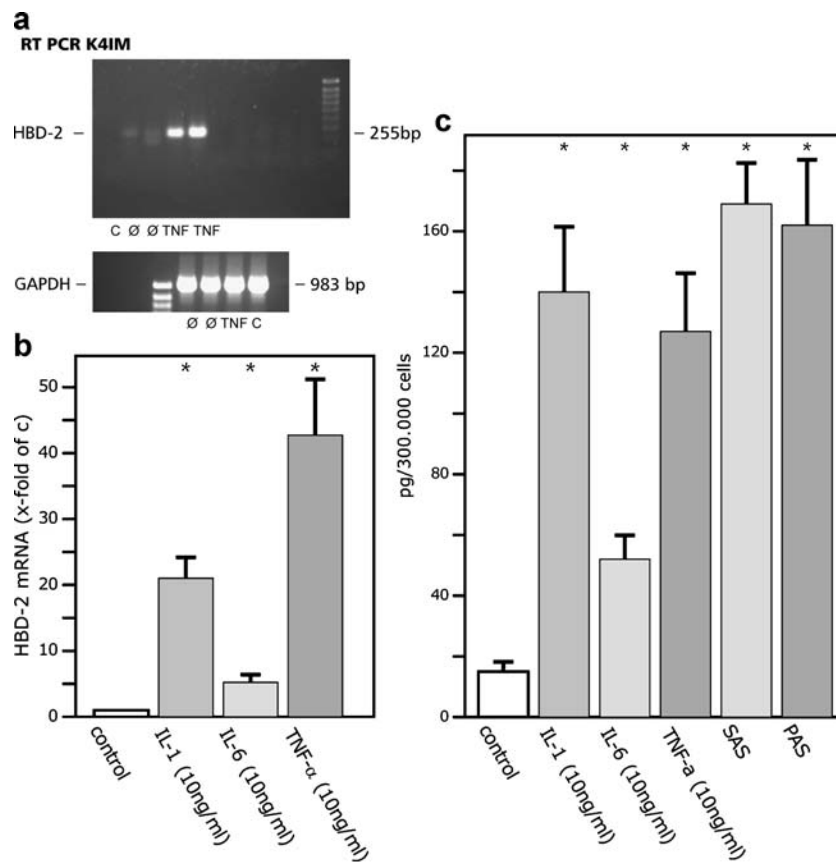

Fig. 2 a–**c** Proinflammatory cytokines such as IL-1, IL-6, or TNF-α and bacterial supernatants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were transcriptional inducers of human β-defensin-2. Proinflammatory cytokines such as IL-1, IL-6, or TNF-α are known to be involved in inflammatory joint disease. To assess their influence on HBD-2 gene expression in cultured K4IM synoviocytes, RT-PCR, real-time RT-PCR, and ELISA experiments were performed. After 6 h of stimulation, TNF-α (10 ng/ml) stimulation resulted in a more than 40-fold increased transcription rate when compared to controls (**a**, **b**). To examine secreted HBD-2 protein levels in cultured K4IM synoviocytes, ELISA experiments were done. After 24 h of stimulation, cell culture supernatants were collected and revealed increased HBD-2 expression in case of IL-1, IL-6, TNF-α, or bacterial exposure (**c**). Interestingly, induction of HBD-2 was not dependent on the specification of the bacteria and did not significantly exceed amounts of cytokine-exposed synoviocytes. Values are the mean ± standard deviation, $*P<0.05$ versus controls. *c* negative control; *Ø* unstimulated cells

affected, but Typ-A and -B-synoviocytes were regarded as the most immunocompetent cells from all. Therefore, we analyzed the production and regulation of the antimicrobial peptide HBD-2 in samples of healthy and infected synovial membranes and evaluated these findings in a model of cultured K4IM synoviocytes after inflammatory or bacterial exposure.

The transcriptional induction of HBD-2 in mesenchymal synovial membrane after contact with proinflammatory cytokines or *S. aureus* is in accordance with previous results on epithelial tissues, which examine the antibacterial role of defensins. Harder et al. [12] were the first who describe the induction of HBD-2 after inflammatory challenge of human skin. Other studies confirmed upregulation in several epithelial tissues such as the lungs or the gastrointestinal or urogenital tract [13, 35–38]. Typical stimulators include the cytokines TNF-α, IL-1/-6, or the gram-negative bacteria *P. aeruginosa* [12, 34, 38, 39]. Many studies failed to observe HBD-2 induction following gram-positive bacterial stimulation, but it is reasonable to propose that AMP induction differs dependent on the examined tissues and the pathogenicity of the used bacteria [12, 39].

The induction of synoviocyte-secreted HBD-2 protein was measured after 24 h of inflammatory or bacterial stimulation. In contrast, AMP secretion of blood cells is induced within a few minutes as a result of storage in cellular granules. The continuous expression of HBD-2 from synoviocytes after inflammatory challenge seems to be more likely a result of a de novo synthesis [10, 11]. Only

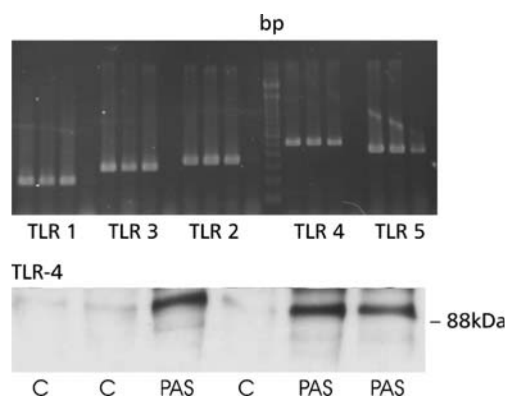

Fig. 3 Toll-like receptors 1–5 were expressed in healthy synovial membranes. To determine putative receptors for bacterial recognition in synovial membranes, RT-PCR and Western blot investigations of Toll-like receptors were performed. RT-PCR revealed transcripts of TLR-1 to -5 in samples of healthy tissues. To assess the inducibility of TLRs after bacterial exposure, primary synoviocytes were challenged by supernatants of *Pseudomonas aeruginosa.* Western blot analysis revealed increased expression of TLR-4 after gram-negative bacterial stimulation, thus suggesting a potential relationship between bacterial recognition via Toll-like receptors and subsequent induction of antimicrobial peptides in synovial membranes. *c* unstimulated cells; *PAS P. aeruginosa*-stimulated cells

two studies have already focused on the expression and regulation of HBD-2 in mesenchymal tissues such as articular cartilage or synovial membranes [26, 32]. Paulsen et al. discovered HBD-2 only in situ in some samples of pyogenic synovial membranes by means of immunohistochemistry [26]. In contrast to our study, all examined tissue samples were tested positive for *S. aureus* colonization and thus may explain their observed incontinuous production of HBD-2.

The induction of HBD-2 in synovial membrane is not merely connected with antibacterial tasks. The secreted protein levels, as measured by ELISA, were at low antibacterial levels, but concomitant expression of cartilage- or neutrophil-released AMPs may increase intra-articular defense levels. In addition to their antimicrobial activity, HBD-2 provides a link to the adaptive immune system by attracting immature dendritic cells and memory T cells via the chemokine receptor CCR-6 [40]. Interestingly, for chemotactic tasks, HBD-2 is needed in much lower concentrations. Recently, two reports describe intra-articular accumulation of AMPs in human joints following abacterial rheumatoid arthritis (RA) [41, 42]. Without immediate threat of bacterial colonization, bactericidal/permeability-increasing protein (BPI) and human neutrophil peptides (HNP-1–3) increased in joint fluid samples of patients with RA. Moreover, they observed a significant correlation between joint destruction and intra-articular accumulation of BPI and HNP, thus suggesting additional tasks of AMPs than the antimicrobial [42]. Previous results of our group may provide an explanation. After co-incubation of chondrocytes or cartilage discs with HBD-3 protein, levels of tissue destructive matrix metalloproteinases raises, and levels of their endogenous inhibitors (tissue inhibitors of metalloproteinases) dropped [31]. Especially, the observed sub-antimicrobial protein levels support our hypothesis that synoviocyte-derived HBD-2 expression following inflammatory exposure may modify migration pattern of blood cells into the joint cavity via CCR-6 receptor or interferes with tissue remodeling processes in articular cartilage.

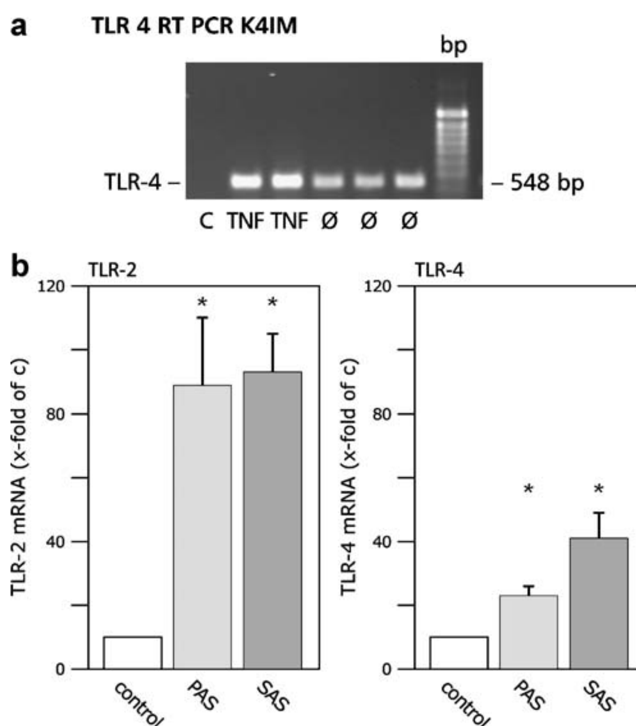

Fig. 4 Toll-like receptor-2 and -4 were induced in cultured K4IM synoviocytes after bacterial stimulation. To determine K4IM synoviocytes as an appropriate in vitro model for studying mechanisms that induce synoviocyte activation, real-time RT-PCR was performed. After 6 h of stimulation, TLR-2 gene expression increased nearly tenfold after gram-positive or -negative bacterial stimulation. Interestingly, TLR-2 is primarily involved in the recognition of gram-positive bacteria in epithelia, but bacterial specification does not influence TLR-2 transcription rate in mesenchymal synoviocytes. The same observation is made in case of TLR-4 real-time analysis. Studies on epithelia suggest that TLR-4 is primarily involved in the recognition of gram-negative bacteria, but mesenchymal synoviocytes induce TLR-4 also after gram-positive bacterial challenge. Values are the mean ± standard deviation, $^{*}P<0.05$ versus controls

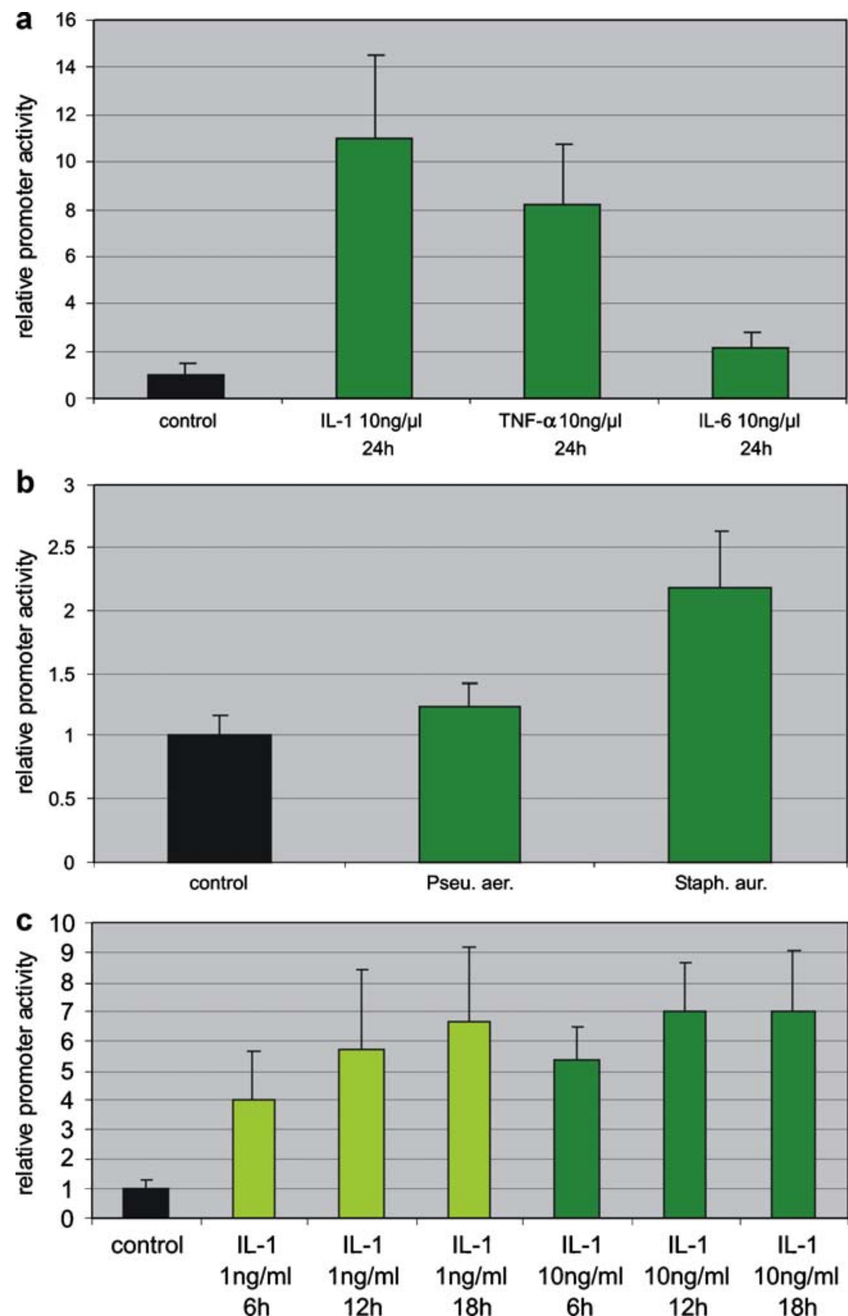

Fig. 5 Dual luciferase assay revealed strong increase of HBD-2 promoter activity due to proinflammatory cytokines or *Staphylococcus aureus* supernatant. IL-1 leads to an 11-fold, TNF-α to an eightfold, and IL-6 to a twofold increase of the HBD-2 promoter activity (**a**). *Pseudomonas aeruginosa* leads to a 1.2-fold and *S. aureus* to a 2.2-fold increase of the HBD-2 promoter activity (**b**). After 12 h, 10 ng/ml IL-1 leads to a maximum of HBD-2 promoter activity (**c**; control=1). All experiments were performed with n=8. *Error bars* indicate SEM. *Significant difference versus control

The receptor, which mediates bacteria- or inflammatory-dependent upregulation of AMP in infectious arthritis, has not yet been characterized. The induction of TLR-2 and -4 in cultured synoviocytes suggest a possible involvement in host AMP production following bacterial stimulation. TLR recognize specific pathogen-associated molecules that are associated with a variety of bacteria, viruses, and fungi [21–23]. Interaction of TLR and bacterial pathogens resulted in an enhanced production of antimicrobial proteins and secretion of proinflammatory cytokines in epithelial tissues, but the role of TLRs in bacteria-infected mesenchymal tissues has to be evaluated in future experiments [21].

Conclusion

Taken together, this is the first report which describes the induction of HBD-2 and the PAMP receptors TLR-2 and -4 in synovial membranes after inflammatory and bacterial exposure and suggests involvement either in innate defense mechanism or in the regulation of the destructive course of septic arthritis.

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Conflicts of interest We declare that we have no conflict of interest.

Authors' contributions

DV, TP, EK, CW, LB, FP, MT, and SL performed the experiments, and BT and AS contributed to the draft manuscript; DV and TP contributed equally to the present work. The manuscript has been read and approved by all authors.

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