Bovine and human cathelicidin cationic host defense peptides similarly suppress transcriptional responses to bacterial lipopolysaccharide

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Abstract: Genomic approaches can be exploited to expose the complexities and conservation of biological systems such as the immune network across various mammalian species. In this study, temporal transcriptional expression profiles were analyzed in human and bovine monocytic cells in response to the TLR-4 agonist, LPS, in the presence or absence of their respective host defense peptides. The cathelicidin peptides, human LL-37 and bovine myeloid antimicrobial peptide-27 (BMAP-27), are homologs, yet they have diverged notably in terms of sequence similarity. In spite of their low sequence similarities, both of these cathelicidin peptides demonstrated potent, antiendotoxin activity in monocytic cells at low, physiologically relevant concentrations. Microarray studies indicated that 10 ng/ml LPS led to the up-regulation of 125 genes in human monocytes, 106 of which were suppressed in the presence of 5 μg/ml of the human peptide LL-37. To confirm and extend these data, temporal transcriptional responses to LPS were assessed in the presence or absence of the species-specific host defense peptides by quantitative real-time PCR. The transcriptional trends of 20 LPS-induced genes were analyzed in bovine and human monocytic cells. These studies demonstrated conserved trends of gene responses in that both peptides were able to profoundly suppress many LPS-induced genes. Consistent with this, the human and bovine peptides suppressed LPS-induced translocation of NF-κB subunits p50 and p65 into the nucleus of monocytic cells. However, there were also distinct differences in responses to LPS and the peptides; for example, treatment with 5 μg/ml BMAP-27 alone tended to influence gene expression (RELA, TNF-α-induced protein 2, MAPK phosphatase 1/dual specificity phosphatase 1, IκBκB, NFκBIL1, TNF receptor-associated factor 2) to a greater extent than did the same amount of human LL-37. We hypothesize that the immunomodulatory effects of the species-specific host defense peptides play a critical role in regulating inflammation and represent an evolutionarily conserved mechanism for maintaining homeostasis, although the sequence divergence of these peptides is substantial. *J. Leukoc. Biol.* 80: 1563–1574; 2006.

Key Words: *endotoxin · inflammation*

INTRODUCTION

Comparative genomics can be used to investigate complex multigenic biological phenomenon, as in principle, essential mechanisms should be conserved in relatively closely related species. Cattle and humans share 72% sequence homology [1] and higher sequence similarity, on average, than that exhibited between human and mouse (F. S. L. Brinkman, unpublished data). Mouse is often used as the animal model of choice to study infectious diseases and the resulting immune responses in mammals. However, as rodents appear to have undergone an accelerated rate of evolutionary divergence relative to many other mammals [2], comparisons between humans and other mammals are becoming more necessary. The comparative genomics of innate immune mechanisms are of great interest, as it has been demonstrated that proteins involved in immunity and host defenses have diverged more rapidly over evolution than many mammalian proteins [3]. Such evolutionarily divergent proteins include a relatively newly described element, the cationic host defense peptides (also termed antimicrobial peptides). For example, the cathelicidin bovine myeloid antimi-

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crobial peptide-27 (BMAP-27) and the human cathelicidin LL-37 share a conserved precursor (pro) peptide sequence and a similar cellular expression pattern, overall cationic amphipathic nature, and α-helical structure but have little sequence similarity in their primary sequences [4]. Conversely, although bacterial LPS vary substantially in compositional chemistry, they are known to be thematically conserved [5], and the innate immune system recognizes these diverse molecules, leading to triggering quite similar responses. Indeed, the process of innate immunity can be triggered by a signal transduction system, which is built to flexibly recognize most LPS molecules, and is initiated by the binding of LPS to a cocomplex of the TLR-4 with myeloid differentiation protein-2 (MD-2), CD-14, and LPS-binding protein (LBP) [6, 7], thereby transmitting signals through certain signal transduction pathways to a variety of transcription factors, the most prominent one of which is NF-κB [8, 9]. Despite the heterogeneity of the immune system and the molecules it recognizes, relatively similar gene responses appear to be induced in different species. In this study, we investigated whether this concept applied to the comparative dynamics of immunomodulation by the divergent, endogenous cationic host defense cathelicidin peptides of human and cattle. Comparisons of the gene expression profiles from various species in response to different stimuli have enormous potential for understanding the extent of conservation of innate immune responses in mammals, as it provides an unbiased and global approach to deciphering the finer details of these responses.

Cathelicidins are cationic, frequently α-helical, amphipathic host defense peptides, which in recent years, have generated a great deal of interest as effector molecules and modulators of innate immunity [10–13]. They are small peptides (<50 amino acids), which are synthesized in a precursor form with a cathelin propeptide sequence, conserved across species [4]. However, the mature peptides vary enormously in sequence, length, and secondary structure. Cathelicidins are expressed as precursor proteins in a variety of cells including myeloid cells, and epithelial cells of many organs, including the skin, oral mucosa, and the gastrointestinal tract, and are found in their proteolytically processed, mature form at mucosal surfaces and in most body secretions, including sweat, breast milk, and saliva [14–18]. Some cathelicidins have antimicrobial activity, but for many peptides, this property is suppressed substantially under physiological salt conditions at the concentrations found at mucosal surfaces [19], and it has been suggested that under these conditions, their main function may be immunomodulatory. In this regard, it has been demonstrated that different peptides have overlapping but often distinct immunomodulatory functions [13, 20], but this aspect is still poorly understood.

Recent evidence has indicated that the sole human cathelicidin, LL-37, is involved in a broad range of immune functions, including up-regulation of chemokines and chemokine receptors in monocytes and epithelial cells, direct recruitment of leukocytes and other cells to the site of infection, and stimulation of mast cells to release histamine and promote diapedesis, promote angiogenesis, modulate dendritic cell development, as well as neutralize bacterial LPS among others [11, 19, 21–25]. Indeed, several of these functions, including its ability to neutralize LPS, have been demonstrated in animal models [26, 27]. Although LL-37 protects animals against certain bacterial infections, its antimicrobial (direct killing) activity is strongly suppressed in the presence of physiologically relevant concentrations of cations such as in tissue culture medium [19]. In contrast to the situation in humans, there are a variety of structurally diverse cathelicidins in cattle [28–30]. The α-helical cathelicidin, BMAP-27, is known to have broad-spectrum, antimicrobial activity in vitro including activity against bacteria [28, 29], viruses [29], and parasites [31]. BMAP-27 has been demonstrated to protect against pathogenic challenge in vivo [29]. However, the involvement of BMAP in innate immune responses has not yet been studied extensively.

As mentioned above, innate immune responses are triggered by recognition of pathogen molecules with conserved features by the family of TLRs, thereby initiating inflammatory responses to pathogenic challenge [32]. There has been speculation and some evidence implicating the role of human cathelicidin LL-37 in maintaining homeostasis, combating pathogenic challenge, and protecting against endotoxaemia, an extreme inflammation-like condition [33–35]. In addition, LL-37 has been demonstrated to modulate the balance of pro- and anti-inflammatory molecules during endotoxin challenge [36]. Therefore, it was of interest to investigate the parallel ability of mammalian cathelicidins to dampen proinflammatory responses.

In this study, we have compared the antiendotoxin effects of human LL-37 and BMAP-27 at physiologically relevant concentrations of less than 1 μg/ml in the face of an endotoxin challenge (10 ng/ml) similar to those observed in septic patients [37]. We further demonstrate overlapping but distinct transcriptional profiles in monocytes from their respective species, whereby the peptides could suppress overall proinflammatory responses in LPS-induced monocytic cells. These studies thus demonstrate the potential of evolutionarily diverged cathelicidins to mediate similar immunomodulatory responses.

MATERIAL AND METHODS

Cell culture and isolation

Human monocytic THP-1 cells were obtained from American Type Culture Collection (Manassas, VA; TIB-202) and were grown in suspension in RPMI 1640 (Gibco™, Invitrogen Corp., San Diego, CA), supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Cultures were maintained at 37°C in a humidified 5% (v/v) CO_2 incubator. THP-1 cells at a density of 1×10^6 cells /ml were treated with 0.3 μg/ml PMA (Sigma-Aldrich, Ontario, Canada.) for 24 h [38], inducing plastic-adherent cells, which were rested further in complete RPMI-1640 medium for an additional 24 h prior to stimulations with various treatments. Purified bovine monocytes (>99.5%) were isolated from the blood of 6- to 8-month-old castrated male calves by MACS® purification using CD14 microbeads (Miltenyi Biotec Inc., Auburn, CA). Purified monocytes were cultured further, 5×10^6 cells/well, in 12-well plates using Aim V medium (Gibco™), supplemented with 5% heat-inactivated FBS (Gibco), 50 μM β-mercaptoethanol, and 2 mM L-glutamine (Sigma-Aldrich). The MACS-purified monocytes were rested for 20 h prior to stimulation.

Stimulants and reagents

Pseudomonas aeruginosa LPS (a known TLR-4 agonist; ref. [6]) was purified to apparent homogeneity from strain H103 using the Darveau-Hancock method as

described previously [39]. Briefly, *P. aeruginosa* H103 was grown overnight in Luria-Bertani broth at 37°C. Cells were collected and washed, and LPS was isolated as described previously. The isolated LPS pellets were back-extracted with a 2:1 chloroform:methanol solution to remove contaminating lipids. The isolated LPS was quantitated using a 3-deoxy-D-manno-octulosonate assay and were resuspended in endotoxin-free water (Sigma-Aldrich). The cationic peptides, human LL- 37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and BMAP-27 (GRFKRFRKKFKKLFKKLSPVIPLLHL) were synthesized by F-moc chemistry at the Nucleic Acid/Protein seq Synthesis Unit at the University of British Columbia (Vancouver, Canada). The synthetic peptides were resuspended in endotoxin-free water (Sigma-Aldrich).

Cell stimulation

Human or bovine monocytic cells were stimulated with a purified LPS (10 ng/ml) in the presence or absence of different concentrations of cathelicidin peptides for the specified time period. All experiments were performed at least three times. In the case of the bovine monocyte experiments, cells isolated from three to five animals were used for each condition.

Screening for proinflammatory cytokine, TNF-α

Untreated cells or cells treated with LPS in the presence or absence of the peptides were centrifuged at 1000 *g* for 5 min followed by 10,000 *g* for 2 min to obtain cell-free tissue-culture supernatant samples. The supernatants were subsequently aliquoted and stored at –20°C until further use. TNF-α secretion was monitored with a species-specific capture ELISA. All assays were performed in triplicate. The concentration of the cytokine was quantified by establishing a standard curve with serial dilutions of the recombinant human TNF-α (eBioscience, San Diego, CA) or bovine TNF-α (Genentech Inc., San Francisco, CA).

RNA extraction and purification

After treatment with the appropriate agents, the plastic adherent THP-1 cells were washed with PBS and then disrupted by scraping in the presence of the lysis buffer (RNeasy Mini kit, Qiagen Inc., Ontario, Canada) with 1% (v/v) β-mercaptoethanol. RNA was isolated from THP-1 cells with the RNeasy Mini kit and treated with RNase-free DNase (Qiagen Inc.), as per the manufacturer's instruction.

The MACS-isolated bovine monocytes were disrupted in the presence of 1 ml Trizol reagent (Gibco), followed by addition of 200 μl chloroform per 1 mL Trizol. The cell suspension was incubated for 3 min at room temperature, after which samples were centrifuged at 12,000 *g* for 10 min at 4°C, and the aqueous phase was collected and precipitated with 500 μL isopropanol per 1 mL Trizol. The solution was incubated further for 5 min at room temperature before being applied to an RNeasy mini-kit (Qiagen Inc.) and centrifuged at 8000 *g* for 15 s, followed by the extraction and purification of RNA and DNase treatment as per the manufacturer's instructions.

Isolated RNA samples were eluted and stored in RNase-free water (Ambion Inc., Austin, TX). RNA concentration, integrity, and purity were assessed using an Agilent 2100 bioanalyzer with RNA 6000 nano kits (Agilent Technologies, Palo Alto, CA).

Hybridization and analysis of DNA microarrays

RNA isolated from human THP-1 cells was reverse-transcribed with incorporation of amino-allyl-uridine 5′-triphosphate using the MessageAmpII™ amplification kit, according to the manufacturer's instructions, and the samples were labeled with monofunctional dyes, Cyanine-3 and Cyanine-5 (Amersham Biosciences Corp., Piscataway, NJ). The samples were purified further using the Mega Clear kit (Ambion Inc.), and the yield and fluorophore incorporation was measured using Lambda 35 UV/VIS fluorimeter (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Microarray slides were printed with the human genome 21 K Array-Ready Oligo Set™ (Qiagen Inc.) at The Jack Bell Research Center (Vancouver, British Columbia, Canada). The slides were prehybridized for 45 min at 48°C in prehybridization buffer containing 5× SSC (Ambion Inc.), 0.1% (w/v) SDS, and 0.2% (w/v) BSA. Equivalent (20 pmol) cyanine-labeled samples from control and treated cells were then mixed and hybridized on the array slides in Ambion SlideHyb™ buffer #2 (Ambion Inc.) for 18 h at 37°C in a hybridization oven. Following hybridization, the slides were washed twice in 1× SSC/0.1% SDS for 5 min at 65°C and then twice in 1× SSC and 0.1× SSC for 3 min each at 42°C. Slides were centrifugated for 5 min at 1000 *g*, dried, and scanned using ScanArray™ Express software/scanner (scanner and software by Packard BioScience BioChip Technologies, Wellesley, MA), and the images were quantified using ImaGene™ (BioDiscovery Inc., El Segundo, CA).

Assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis was carried out with ArrayPipe (Version 1.6), a web-based, semi-automated software specifically designed for processing of microarray data [40] (www.pathogenomics.ca/arraypipe). Data analysis was performed by flagging of markers, subgrid-wise background correction using the median of the lower 10% foreground intensity as an estimate for the background noise, data-shifting to rescue negative spots, printTip LOESS normalization, merging of technical replicates, two-sided, one-sample Student's *t*-test on the $\log_2$ ratios within each treatment group, and averaging of biological replicates to yield overall fold changes for each treatment group.

Gene validation by quantitative real-time RT-PCR (qRT-PCR)

Validation of the microarray results and further temporal assessment of transcriptional profile were performed by qRT-PCR for the selected genes of interest in human and bovine cell types. Gene candidates selected from the human microarray analysis were screened in bovine monocytes by using primers based on the bovine orthologs. The orthologs were determined using reciprocal best BLAST hit analysis between human (Ensembl Version 31.35d) and bovine (The Institute for Genomic Research gene indices Version 11) genomic data. The trends of gene expression were compared between LPS-stimulated human and bovine monocytic cell populations in the presence or absence of the species-specific cathelicidin over a time period of 1–24 h. qRT-PCR was performed using Invitrogen's SuperScript™ III Platinum® two-step qRT-PCR kit with SYBR® Green on the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) for human cells and on the Bio-Rad (Hercules, CA) iCycler for bovine cells. Briefly, 1 μg total RNA was incubated with 5 μL 2× RT reaction mix and 0.5 μL RT, followed by 50 min incubation at 42°C. The reaction was terminated by incubating for 5 min at 85°C. The cDNA mixture was then incubated for 30 min at 37°C in the presence of RNase H. The PCR reaction was carried out as per the manufacturer's instructions (Invitrogen Corp.) using a mix of Platinum® SYBR® Green qRT-PCR Super-Mix UDG, the template cDNA, 10 mM of the primer mix, and DNase-free H_2O up to 12.5 μL total vol/well. Cycling conditions were carried out as indicated by the Invitrogen's SuperScript™ III Platinum® two-step qRT-PCR kit with SYBR® Green. A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes for the genes of interest were performed after normalization with the species-specific endogenous control GAPDH and using the comparative threshold cycle (Ct) method [41]. The primers used for qRT-PCR in bovine and human species are listed in **Table 1**. Amplicons from bovine samples were sequenced in-house to validate primer specificity and amplicon identity.

Assay for translocation of NF-κB subunits using nuclear extracts

Human monocytic THP-1 cells (3×10^6) were seeded into 60 mm^2 petri dishes (VWR International, Ontario, Canada) and were stimulated with LPS (10 ng/ml) in the presence or absence of human LL-37 or BMAP-27 (5 μg/ml) for 60 min. The cells were treated by simultaneous addition of LPS with the peptides or by pretreatment of the cells with LPS for 30 min, followed by the addition of peptides for an additional 60 min. Cells were subsequently treated with Versene for 10 min at 37°C in 5% CO_2 (to detach adherent cells) and then washed twice with ice-cold PBS. Cytoplasmic and nuclear extracts were isolated using NE-PER® nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The protein concentration of the extracts was quantified using a bicinchoninic acid protein assay (Pierce Biotechnology), and the extracts were stored at –80°C until further use.

Equivalent nuclear extracts (5 μg) were analyzed for NF-κB subunits p50 or p65 by StressXpress NF-κB p50 or p65 ELISA kits (Stressgen Bioreagents, Victoria, British Columbia, Canada) according to the manufacturer's instructions. Luminescence was detected with SpectraFluor Plus Multifunction microplate reader (Tecan Systems Inc., San Jose, CA).

TABLE 1. Human and Bovine Primers Used for qRT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
	Human primers	
CCL4	CTTTTCTTACACCGCGAGGAA	GCAGAGGCTGCTGGTCTCAT
CCL20	TGACTGCTGTCTTGGATACACAGA	TGATAGCATTGATGTCACAGCCT
CXCL1	GCCAGTGCTTGCAGACCCT	GGCTATGACTTCGGTTTGGG
IL-8	GACCACACTGCGCCAACAC	CTTCTCCACAACCCTCTGCAC
IL-10	GGTTGCCAAGCCTTGTCTGA	AGGGAGTTCACATGCGCCT
TNF-α	TGGAGAAGGGTGACCGACTC	TCCTCACAGGGCAATGATCC
TNFAIP2	CTACCAGCGCGCCTTTAATG	TCCGGAAGGACAGGCAGTT
TNFAIP3	CTGCCCAGGAATGCTACAGATAC	CAGGGTCACCAAGGGTACAAA
TNIP3	TGAAAGAAAGGTAGCAGAGCTGAA	CCGCGTGCTGAGGAATCT
BIRC3	AAAGCGCCAACACGTTTGA	AGGAACCCCAGCAGGAAAAG
SOCS1	TCCCCCTGGTTGTTGTAGCA	TTGTGCAAAGATACTGGGTATATGTAAA
HIF1-α	AGCCAGATCTCGGCGAAGT	CAGAGGCCTTATCAAGATGCG
HCK	GTCCTGTGTACGTGCCGGA	GATGTCCTCAGAGCCTGCCT
SOD2	GACCTGCCCTACGACTACGG	TTCAGGTTGTTCACGTAGGCC
TRAF2	CTGCCCCAAGTTCCCCTTAA	GGACTCGACACTTGCCACAA
NFκB1	CTTAGGAGGGAGAGCCCACC	TTGTTCAGGCCTTCCCAAAT
REL A	TAGGAAAGGACTGCCGGGAT	CCGCTTCTTCACACACTGGA
NFκBIL1	GAGCGGGAATGGAGACAGAA	GGTTCCTGGGTTTCATGGG
NFκBIA	GGTGAAGGGAGACCTGGCTT	GTGCCTCAGCAATTTCTGGC
IκBκB	GTGGAAGCCCGGATAGCAT	CTTGGCTGGCTCAGGTAAGC
	Bovine primers	
CCL4	GCTCCGGGTGA-ATCTCTCCT	CTGAGAGCGCTGGAGAACAGA
CCL20	GAAGCAAGCAACTTCGACTGC	TCACAGGCTTCATTGGCCA
CXCL1	ATGCTGTTCCTGCTCCTG	TGTCACCTTCACGCTCTG
IL-8	GAGAGTGGGCCACACTGTG	CAACCTTCTGCACCCACTTTTC
IL-10	CCGCCCTCTGATTTCTCTTG	CGGCTCCCTGGTTTCTCTTC
TNF-α	CCCCTGGAGATAACCTCCCA	CAGACGGGAGACAGGAGAGC
TNFAIP2	AAGCCCTTGCTGTTGGAAG	CCCTGTAATTGCGCAGCTGT
TNFAIP3	GGGACAATGTGCTTCCCTTC	AACCTCTG-TCTGTCCGTGGC
TNIP3	AAATGGAGTGCAGCCGTGAG	TCGGTCTGATCGCTCCTTTT
BIRC3	GGAGAAAGAAAGAGCAACTGAGGA	TTGGAAGCACACAAGTCAGATGT
SOCS1	TAAACATGAAGAGGTAGGAGGTACGA	CTGGTGCTCCCTCTGGGTC
HIF1-α	TTCCATCTCCT CCCCACGTA	TTTTCCTGCTCTGTTGGGTGA
HCK	TTCTCAGCCCAGATTGCAGA	ACCAGGGATGCAGACACCAA
SOD2	CCGAGGAGAAGTACCGGGAG	GTGATTGATATGGCCCCCAC
TRAF2	TGCTCCCCTGCCGACAT	TGAAACTTCTCACGGGCGAT
NFκB1	TTTCAACCGGAGATGCCACT	GGGCCTTCACACACGTAACG
REL A	CTGGAGAGAGGAGCACGGAC	GTCCTTGGTGACCAGGGAGA
NFκBIL1	GGAGAGCTGGAGGACGAGTG	CGATCTGACCAGGCTGAGAAG
NFκBIA	GGGAGACCTGGCCTTCCTC	CCAGAAGTGCCTCAGCGATT
IκBκB	CGGCCATGATGAATCTGCT	TAAAGAAGTCCAGCTTGGCCTT

TNFAIP2/3, TNF-α-induced protein 2/3; SOCS1, suppressor of cytokine signaling 1; HIF-1α, hypoxia-inducible factor-1α; SOD2, superoxide dismutase 2; TRAF2, TNF receptor-associated factor 2; TNIP, TNFAIP3-interacting protein 3; BIRC3, baculoviral IAP repeat-containing 3; HCK, hemoietic cell kinase; REL A, NF-κB subunit.

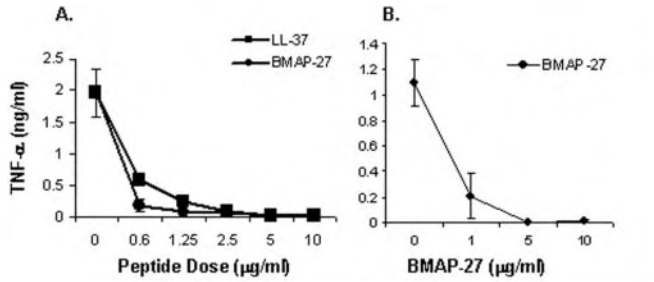
Statistical analysis

A two-sided, one-sample Student's *t*-test on the $\log_2$ ratios was performed within each treatment group for the DNA microarray analyses, and $P \leq 0.05$ was considered statistically significant. The details of the statistical approaches used are outlined at www.pathogenomics.ca/arraypipe [41]. Values shown are expressed as mean ± 1 SD of the mean.

RESULTS

Human and bovine cathelicidins exhibit antiendotoxin properties at low concentrations

Previous studies have indicated that LL-37 could selectively modulate inflammatory responses induced by high doses of LPS (100 ng/ml) in human monocytic cells [36]. Under these circumstances, relatively high concentrations of LL-37 were applied to study transcriptional responses, equivalent to those found in inflamed tissues [42]. In this study, we used a level of endotoxin similar to that found in septic patients (10 ng/ml) [37]. To compare the antiendotoxin activity of human and bovine cathelicidins, the concentration of the proinflammatory cytokine TNF-α was measured by ELISA in the tissue-culture supernatants of monocytic cells stimulated by LPS (10 ng/ml) for 4 h in the presence and absence of human LL-37 or BMAP-27. Similar dose-dependent inhibition of LPS-induced TNF-α production was observed with LL-37 and BMAP-27 using the human monocytic THP-1 cell line (**Fig. 1A**). Human LL-37 and BMAP-27 suppressed LPS-induced TNF-α by



%Inhibition of LPS-induced TNF-α in THP-1 cells		
Peptide Dose (μg/ml)	LL-37	BMAP-27
0.6	70 (+3.1)	91 (+4)
1.25	87 (+2)	95 (+2.96)
2.5	95 (+1.3)	97 (+0.96)
5	99 (+0.89)	99 (+0.16)
10	99 (±0.21)	99 (±0.12

%Inhibition of LPS-induced TNF-α in Bovine monocytes	
Peptide Dose (μg/ml)	BMAP-27
1	81 (+10.4)
5	99 (+0.1)
10	99 (+0.8)

Fig. 1. Human and bovine cathelicidins exhibit antiendotoxin properties at low concentrations. The concentration of the proinflammatory cytokine TNF-α was monitored by ELISA in the tissue-culture supernatants of cells stimulated by 10 ng/ml LPS in the presence of varying concentrations of human LL-37 or BMAP-27. The results are an average (±1 SD) of three independent experiments. (A) Human THP-1 cells were stimulated with LPS for 4 h in the presence of increasing concentrations of human LL-37 or BMAP-27. (B) CD14 monocytes isolated from bovine PBMC were stimulated with LPS for 24 h in the presence of increasing concentrations of BMAP-27.

>70% at as low as 0.6 μg/ml and by more than 99% at 5 μg/ml. Antiendotoxin activity was also assessed in bovine monocytes isolated from PBMC, stimulated with 10 ng/ml LPS for 24 h in the presence of increasing concentrations of BMAP-27. LPS-induced TNF-α production in bovine monocytes was decreased by more than 80% with 1 μg/ml BMAP-27 and reduced to background levels (≥99%) by treatment with 5 μg/ml BMAP-27 (Fig. 1B), in agreement with studies with human cells (Fig. 1A). A similar antiendotoxin effect for LL-37 was also observed with human PBMC (data not shown). Thus, essentially the same antiendotoxic activities were observed for these quite diverse peptides in the human and bovine monocytic cellular systems. Subsequent studies to evaluate the comparative antiendotoxin properties of LL-37 and BMAP-27 were performed using 10 ng/ml LPS and 5 μg/ml of the respective peptides. In the case of LL-37, it has been demonstrated that the physiological levels in the human lung range from 2 to 5 μg/ml [42].

Retention of antiendotoxin activity after delayed addition of cathelicidins

To better understand the kinetics of antiendotoxin activity for the two peptides, human THP-1 cells were stimulated with LPS (10 ng/ml) for 30 min, followed by the delayed addition of human LL-37 or BMAP-27 (5 μg/ml). Tissue culture supernatants were subsequently collected after 1, 2, 4, and 24 h of incubation and screened for proinflammatory responses by measuring TNF-α by ELISA. The peptides had no effect on the rather modest LPS-induced production of TNF-α at the 1- and 2-h time-points in human monocytes, but significant antiendotoxin activity of the peptides was observed after 4 and 24 h of stimulation (**Fig. 2A**), with ≥60% inhibition of LPS-induced TNF-α production with delayed addition (Fig. 2A), as opposed to 99% inhibition when LL-37 was added at the same time as LPS (Fig. 1). Similarly in bovine monocytes, the addition of 5 μg/ml BMAP-27, 30 min after LPS, was adequate to quench the LPS-induced TNF-α response when monitored after 20 h of stimulation (data not shown). In addition, the host defense peptides significantly retained their antiendotoxin activity when added 30 min after LPS stimulation, even after removal of LPS from the extracellular media (Fig. 2B). LPS-induced TNF-α was reduced by 57 ± 10% with LL-37 ($P<0.001$) and by 84 ± 4% with BAMP-27 ($P<0.001$). It is to be noted that LPS gets internalized within the cytoplasm of monocytic cells within 10–30 min of stimulation (**Fig. 3**), indicating that LPS-induced responses can be triggered at early time-points. These results together indicate that a concentration of 5 μg/ml of each host defense peptide was adequate to quench TNF-α secretion, even when added after LPS stimulation. LPS is internalized by human monocytic cells within 10–30 min of stimulation. Nevertheless, the LPS-induced, proinflammatory

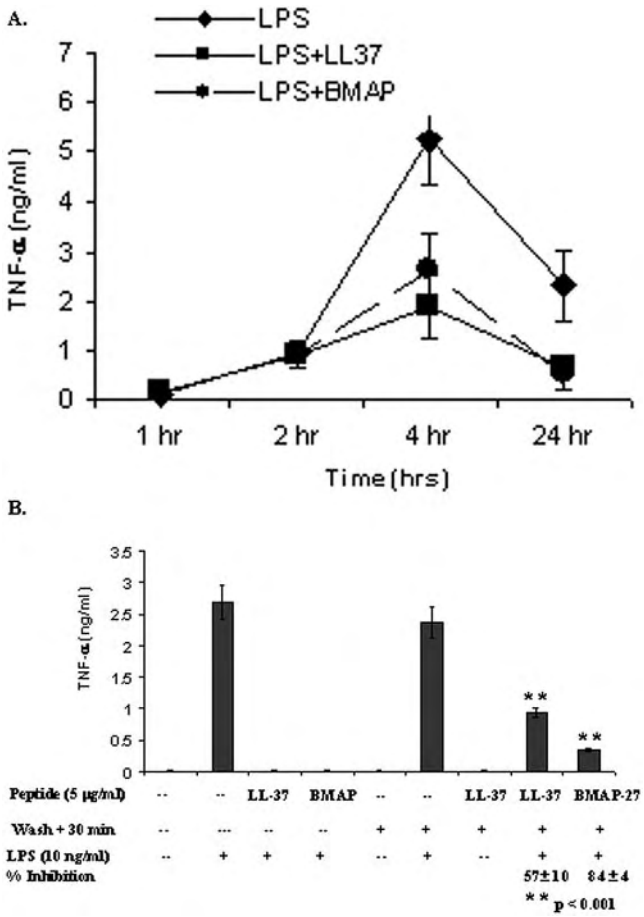


Fig. 2. Retention of antiendotoxin activity after delayed addition of cathelicidins. (A) Human THP-1 cells were stimulated with LPS (10 ng/ml) for 30 min, followed by the addition of human LL-37 or BMAP-27 (5 μg/ml). (B) Human THP-1 cells were stimulated with LPS (10 ng/ml) for 30 min, followed by removal of LPS from the extracellucar medium by washing the cells with RPMI media (×3), followed by the addition of human LL-37 or BMAP-27 (5 μg/ml). Tissue-culture supernatants were collected after 1, 2, 4, and 24 h (*x*-axis) of incubation and screened for TNF-α production by ELISA.

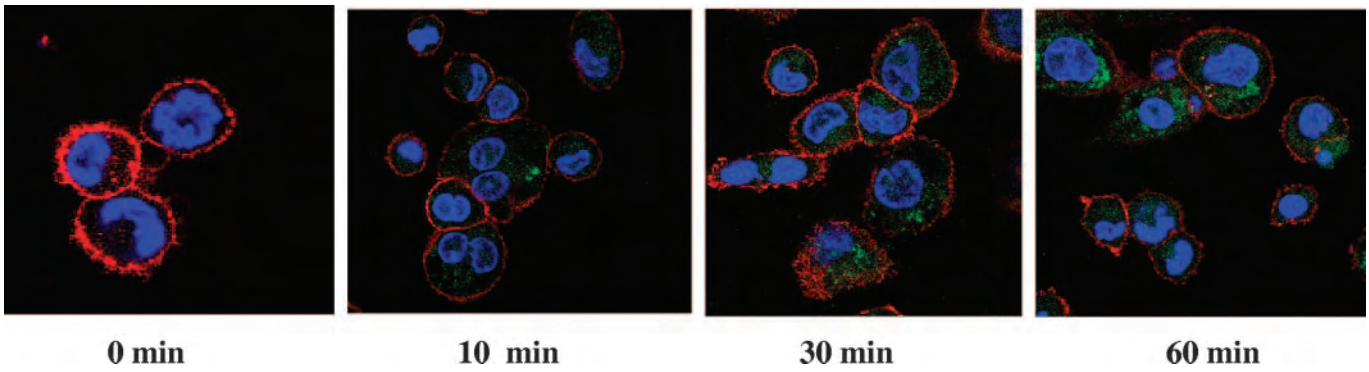


Fig. 3. Internalization of LPS in human monocytic cells within 10–30 min of stimulation. THP-1 cells were seeded at a density of 5×10^4 cells per coverslip in complete RPMI and differentiated with PMA. LPS-Alexa 488 (green color; Molecular Probes, Eugene, OR) was incubated with cells for 10 min^{-1} h at 37°C under 5% CO_2. Following treatment, coverslips were washed with PBS and fixed with 4% paraformaldehyde. The coverslips were washed extensively after fixing, and the cells were permeabilized by using 0.1% Triton X-100 in PBS and probed with streptavidin (Molecular Probes). Cells were washed extensively with PBS and then probed with Alexa-conjugated phalloidin (Molecular Probes) to detect actin (red). Coverslips were mounted in VectaShield with 4′,6′-diamidino-2-phenylindole to stain (blue) for host cell DNA (Vector Laboratories, Burlingame, CA). Coverslips were viewed by using a Nikon confocal microscope.

response could be suppressed when host defense peptides were added 30 min after stimulation with LPS and its subsequent removal from the extracellular medium. Therefore, the ability of these peptides to reduce LPS-induced, proinflammatory responses is hypothesized to be partly independent of LPS binding

Microarray transcriptional profiling with low doses of LPS and LL-37 in human monocytic cells

We have previously investigated the transcriptional profile of human monocytic THP-1 cells using higher doses of LPS (100 ng/ml) and LL-37 (20 μg/ml) [36], concentrations that are equivalent to those found at sites of persistent infection complicated by chronic inflammation. To serve as a basis for comparing LL-37 with BMAP-27 at more physiological concentrations, we repeated the DNA microarray experiments stimulating human monocytic THP-1 cells with a lower dose of LPS (10 ng/ml) in the presence or absence of LL-37 (5 μg/ml). These transcriptional analyses of THP-1 monocytes, using human 21 K oligo-based DNA microarrays, were done at a single time-point (4 h), using three independent biological experiments, each with two technical repeats, comparing treated and unstimulated cells. Statistically significant, differentially expressed genes were selected from each treatment type as those with a fold change of ≥1.5 with a Student's *t*-test *P* value of ≤0.05. The microarray data have been deposited into ArrayExpress under accession number E-FPMI-6. LPS alone caused a relatively muted response compared with that observed using the higher LPS dose [36]. In this study, 125 genes were differentially expressed in LPS-stimulated cells (Supplemental Table 1). Of these, 106 genes were suppressed to baseline expression in the presence of LL-37, and only 19 genes (16%) were identified as still being up-regulated in cells stimulated with LPS in the presence of LL-37 (Supplemental Table 2). The presence of low, physiological concentrations of LL-37, therefore, had an overall strong, suppressive effect on proinflammatory responses induced by LPS in human monocytic cells. From these microarray studies, 20 LPS-induced genes (those that were altered significantly in the presence of the host defense peptide) were selected for further confirmation and investigation using qRT-PCR.

Trends of temporal transcriptional response elicited by endogenous cathelicidins in LPS-induced monocytes are similar in bovine and human species

The temporal expression profiles of the selected genes of interest were analyzed by qRT-PCR in human and bovine monocytic cells in the presence of LPS alone, LL-37, and BMAP-27, respectively, or the LPS and species-specific peptide combined over a time period of 1–24 h (**Fig. 4**). Fold changes in the expression of the genes of interest were calculated relative to expression in unstimulated control cells as described previously [41].

The overall trend of endotoxin-induced responses in human and bovine cells was similar, with a few obvious differences between the two species (Fig. 4; **Table 2**). In addition, the overall trend of transcriptional responses observed on LPS stimulation in the presence and absence of human cathelicidin LL-37 in human monocytic cell line THP-1 (Fig. 4) was similar to that observed in human PBMC (data not shown).

In the presence of endotoxin, the genes encoding the proinflammatory molecules TNF-α, HIF1-α, and NF-κB1 were up-regulated by ten- to 50-fold in human and bovine cells. Although other proinflammatory responses such as TNFAIP2 and IL-8 were also up-regulated in the species, the response was more pronounced in human monocytes than in bovine monocytes, and the up-regulation in human cells was 35- to 60-fold, whereas bovine cells demonstrated less than tenfold up-regulation. Similarly, endotoxin-induced chemoattractants were differentially induced. For example, although CCL20 was up-regulated in the presence of LPS by more than 130-fold in human and bovine cells, CCL4 and CXCL1 were highly up-regulated in human cells by between 80- and 200-fold but in contrast, were up-

TABLE 2. Comparison of Relative Expression of Selected Genes in Human and Bovine Monocytic Cells by qRT-PCR

		Relative expression by qRT-PCR at 4 h			
		Human		Bovine	
Gene name	Brief gene description	LPS	LPS + LL-37	LPS	LPS + BMAP-27
NFκB1	Involved in generation of p50 by a cotranslational processing.	8.2	2 (1.7)[a]	7	1.8 (1.8)[a]
NFκBIA	Inhibits NF-κB by complexing with and trapping it in the cytoplasm.	13.6	2.5	4.6	2.6
NFκBIL1	Ankyrin; tyrosine-specific protein phosphatase and dual specificity protein phosphatase.	1	1	2.6	2.6 (2.8)[a]
RELA	NF-κ-B/Rel/dorsal family; generation of p65.	1.5	1	3.4	2.3 (3.1)[a]
IκBκB	Phosphorylates inhibitors of NF-κB, thus leading to the dissociation of the inhibitor/NF-κB complex.	1.2	1.1	5.6	3.3 (3)[a]
TNF-α	Cytokine secreted by macrophages is a potent pyrogen. Under certain conditions, it can stimulate cell proliferation and induce cell differentiation.	56	5	47	4
TNFAIP2	Mediator of inflammation and angiogenesis.	60	4.4	8	2 (2.6)[a]
TNFAIP3	Inhibits TNF-induced, NF-κB-dependent gene expression by interfering with repeat-induced point mutation- or TRAF2-mediated transactivation signal.	14	3.5 (1.6)[a]	7.9	1.9
TNIP3	TNFAIP3-interacting protein 3.	47	1.1	50.4	3.3
IL-8	IL-8 is a chemotactic factor also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus.	35	2.8 (1.6)[a]	4	1.2
IL-10	Inhibits the synthesis of a number of cytokines, including IFN-γ, IL-2, IL-3, TNF, and GM-CSF, produced by activated macrophages and by helper T cells.	21	1.2 (2)[a]	11.4	3.2
CCL4	Monokine with inflammatory and chemokinetic properties.	226	7.7	7.6	1
CCL20	Chemotactic factor that attracts lymphocytes and slightly, neutrophils but not monocytes. May be involved in formation and function of the mucosal lymphoid tissues by attracting lymphocytes and dendritic cells towards epithelial cells.	145	6	139	2.8
CXCL1	Acts as a scavenger receptor on macrophages, which specifically binds to oxidized low-density lipoprotein. Induces a strong chemotactic response. Induces calcium mobilization.	88	4.9 (1.7)[a]	26.9	1.8
BIRC3	Apoptotic suppressor. Interacts with TRAF1 and TRAF2 to form heteromeric complex, which is then recruited to the tumor necrosis factor receptor 2 (TNFR2).	165	7.5	20.5	1.4
SOCS1	SOCS1 is involved in negative regulation of cytokines that signal through the JAK/STAT3 pathway. Appears to be a major regulator of signaling by IL-6.	4.5	1	17.6	2.9
MKP1/DUSP1	Dual-specificity phosphatase that dephosphorylates MAP-κ ERK2 on Thr-183 and Tyr-185.	2.5	1.1	6.4	2.3 (2.1)[a]
TRAF2	Adapter protein and signal transducer that links members of the TNFR family to different signaling pathways by association with the receptor cytoplasmic domain and kinases. Mediates activation of NF-κB and JNK.	1.6	1.02	5.55	3.9 (2.6)[a]
HIF1-α	Functions as a master transcriptional regulator of the adaptive response to hypoxia. Under hypoxic conditions, activates the transcription of over 40 genes.	2	0.7	6.3	2 (1.6)[a]
HCK	May serve as part of a signaling pathway coupling the Fc receptor to the activation of the respiratory burst. May also contribute to neutrophil migration and may regulate the degranulation process of neutrophils.	4.9	1.4	6.8	2

[a] Numbers in brackets are values of peptide alone when the fold change relative to unstimulated cells is >1.5.

regulated to a substantially lesser extent of between eight- and 25-fold in bovine monocytes. The expression of dual-phosphatase MKP1 and the transcription factor TRAF2 was up-regulated by LPS in human and bovine monocytic cells. It is interesting that the major transcription factor NF-κB subunits, NFκB1 (p50) and RELA (p65), were up-regulated in human and bovine monocytes, but in contrast, the NF-κB antagonist IκBκB was up-regulated fivefold in bovine cells, whereas its expression remained unchanged in human monocytic cells in the presence of LPS.

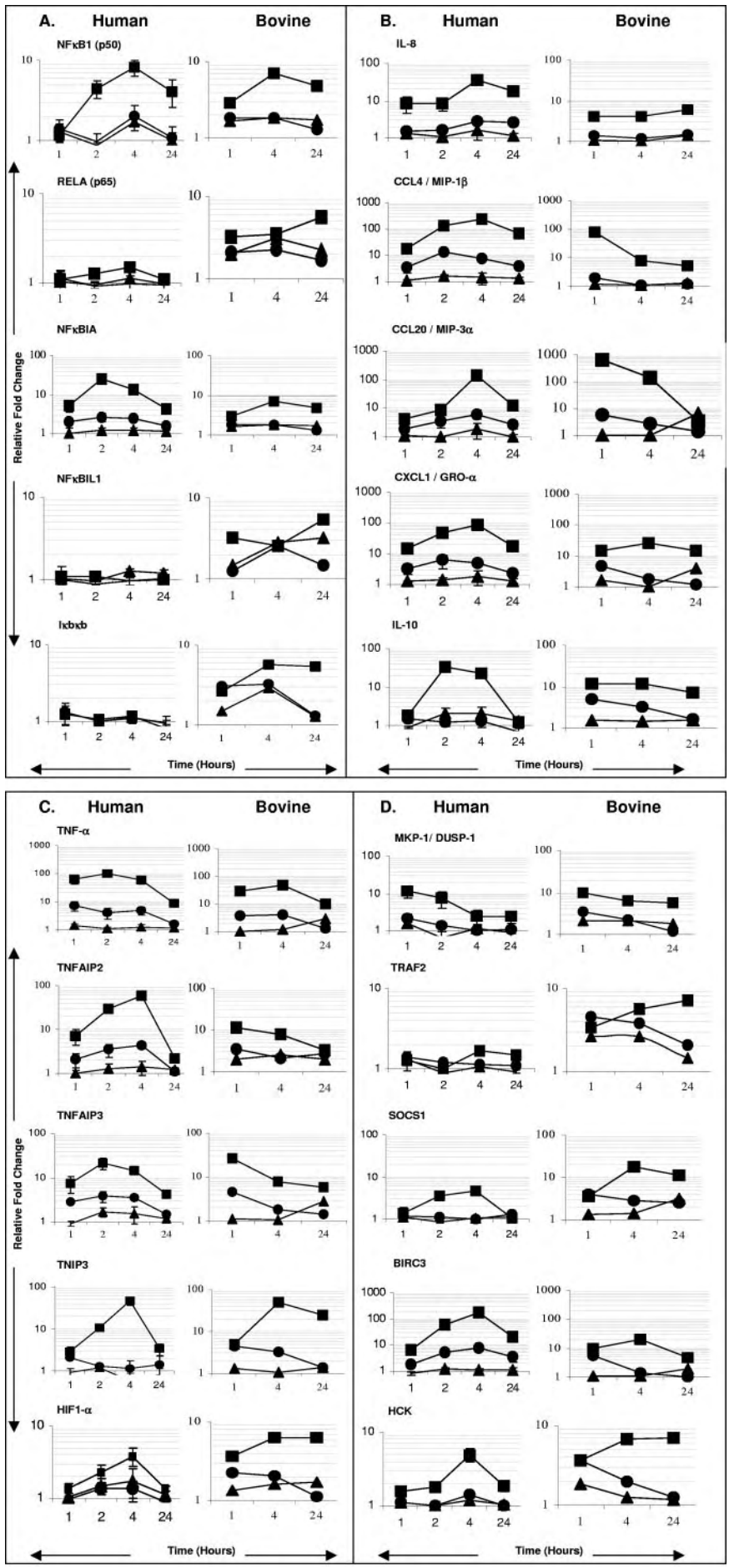


Fig. 4. Trends of temporal transcriptional response elicited by endogenous cathelicidins in LPS-induced monocytes are similar in bovine and human species. Trends of transcriptional response of genes of interest were validated by qRT-PCR over a time period of 1–24 h (*x*-axis) in human and bovine systems. Gene expression in LPS-stimulated cells (■), cells treated with the peptide alone (▲), or cells treated with a combination of LPS and peptide (●) was analyzed by qRT-PCR. Results shown are the mean ± SE of three independent experiments. Relative fold changes (*y*-axis, log scale) for each gene were normalized to human GAPDH or bovine β-actin, respectively. Fold changes are calculated by the Ct method and are relative to the gene expression in unstimulated cells (normalized to 1). (A) NF-κB and related genes; (B) chemokines and ILs; (C) TNF-α and related genes; (D) transcription factors and other regulatory genes. GRO-α, Growth-related oncogene-α; MKP-1, MAPK phosphatase 1; DUSP-1, dual specificity phosphatase 1.

The transcriptional responses elicited by the species-specific cathelicidins acting independently on human and bovine monocytic cells were clearly different. Human LL-37 alone at 5 μg/ml induced insignificant responses in the tested genes relative to the unstimulated control cells. In contrast, bovine 5 μg/ml BMAP-27 induced the statistically significant up-regulation of several genes (e.g., NFKB1/p50, RELA, TNFAIP2, IL-10, MKP1/DUSP1, IκBκB, NFκBIL1, TRAF2) by approximately twofold (Table 2). In spite of these differences, both cathelicidin peptides had similar effects on LPS-induced transcriptional responses in human cells and bovine cells (Fig. 3).

Human LL-37 and BMAP-27 significantly reduced the expression of LPS-induced genes encoding proinflammatory molecules (TNF-α, TNFAIP2, NFκB1, HIF1-α) by 65–93% within 4 h of stimulation (Fig. 4; Table 2). Similarly, the expression of the LPS-induced chemokines and cytokines CCL4, CCL20, CXCL1, IL-10, and IL-8 were reduced substantially (eight- to 40-fold) by the presence of the species-specific cathelicidins (Fig. 4; Table 2). However, it is worth noting that the LPS-induced expression of the chemoattractants was not neutralized completely by the peptides, indicating the partial maintenance of the expression of genes required for cell recruitment in both the cell types, consistent with the known effects of host defense peptides on direct and indirect chemoattraction of immune cells [10, 13, 19–21].

The expression of LPS-induced negative regulators of NF-κB was also not neutralized completely by the cathelicidin peptides in both species; however, there appeared to be different regulators expressed in the two species (Fig. 4; Table 2). Although the expression of the LPS-induced negative regulators of NF-κB, such as NFκBIA and TNFAIP3, was decreased by four- to fivefold in human cells, these molecules still remained up-regulated in the presence of LL-37. Similarly, LPS-induced expression of SOCS1, a suppressor of cytokine signaling, was decreased by five- to sixfold in human and bovine cells but was not neutralized completely in bovine monocytes. In bovine monocytes, the expression of the LPS-induced negative regulator of NF-κB, NFκBIL1, was not decreased and instead remained up-regulated to the same extent in the presence of BMAP-27. In human and bovine cell types, LPS-induced gene expression of NFκB1 (encoding for NF-κB subunit p50) was inhibited by ≥75%, and the expression of RELA (NF-κB subunit p65) was reduced by ≥33% in the presence of the respective endogenous cathelicidins (Table 2).

The overall transcriptional profiles elicited by LPS in the presence and absence of the species-specific cathelicidins in human and bovine monocytic cells were similar. In contrast, gene expression profiles elicited by the peptides themselves (without LPS) were different between the two mammalian species.

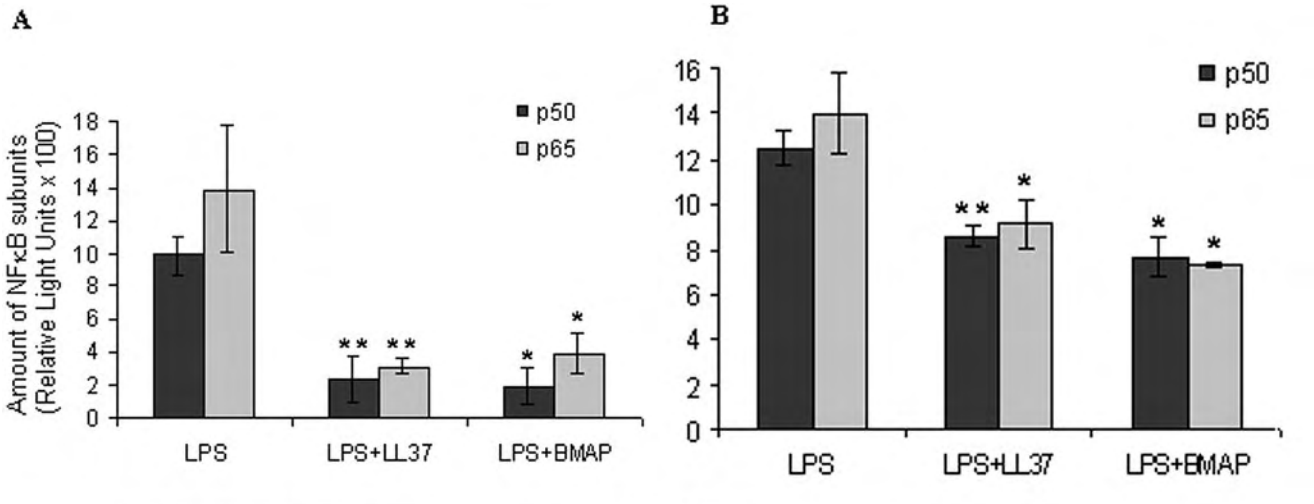
Cathelicidins LL-37 and BMAP-27 suppressed LPS-induced translocation of NF-κB subunits p50 and p65

LPS-induced activation of NF-κB, involving the degradation of cytosolic IκB and migration of NF-κB subunits into the nucleus, is known to be mediated through the TLR-4 pathway [6, 8, 9, 43, 44]. NF-κB and particularly, the NF-κB heterodimer, comprised of p50 and p65 subunits, play a central role in innate immune responses to pathogenic challenge and in the onset of sepsis triggered by endotoxin [6–9, 45]. As the LPS-induced gene expression of NFκB1 and RelA was suppressed significantly by both peptides, the effect of these cathelicidins on LPS-induced nuclear translocation of p50 and p65 was analyzed in this study. Both cathelicidins exhibited an ability to suppress LPS-induced translocation of NF-κB subunits p50 and p65 when added simultaneously with LPS (by 71–79%; **Fig. 5A**), as well as when added after 30 min of LPS stimulation (by 31–47%; Fig. 5B), but the extent of suppression of the subunits differed in the two cases (Fig. 5). This observation clearly indicates that the ability of cathelicidins to suppress the endotoxin-induced activation of NF-κB subunits is conserved across species.

DISCUSSION

Cross-species comparisons of gene expression can be a powerful tool to evaluate evolutionarily conserved, biological mechanisms. Host defense peptides are critical effectors and modulators of innate immunity, which are widely distributed in nature from insects to mammals, although they are extraordinarily diverse in terms of their sequences. It is thus of considerable interest to determine if their roles in innate immunity overlap or even correspond despite the considerable differences in structure and sequence between peptides within and between species. In this manuscript, we have obtained data indicating considerable similarities in the mechanisms of antiendotoxin activity of endogenous cathelicidins in two different mammalian species.

The LPS response in mammals is mediated primarily by the TLR-4-to-NF-κB pathway [6, 8, 43, 44]. Human LL-37 has been demonstrated to selectively alter the LPS-induced TLR-to-NF-κB pathway when present at comparatively high doses of 20 μg/ml [36]. In the present study, it was interesting to observe that modest levels of human LL-37 and BMAP-27 exhibited similar antiendotoxin effects in significantly suppressing proinflammatory TNF-α production in the presence of LPS/endotoxin amounts comparable with those found in septic patients [37]. As LL-37 is found at mucosal surfaces and in the lung at concentrations of 2–5 μg/ml [19, 42], it is clearly antiendotoxic at physiologically relevant concentrations and salt conditions, although the antibacterial activity of LL-37 is blocked completely in the presence of tissue-culture medium [19]. The overall proinflammatory, transcriptional response induced by LPS was suppressed by 70–98% within 4 h in the presence of human LL-37 and BMAP-27 in their respective species (Fig. 4; Table 2). Similarly, the expression of various LPS-induced chemokines was reduced substantially but not neutralized completely by the presence of the species-specific cathelicidins. This indicated that both peptides balanced the suppression of endotoxin-mediated inflammatory responses by partially maintaining the expression of certain genes that are required for cell recruitment to combat pathogenic challenge. Consistent with these studies, it has been demonstrated previously that LL-37 at modest concentrations can itself up-regulate chemokines such as IL-8 [21], and at somewhat higher concentrations, it can act as a chemoattractant for monocytes,



%Inhibition of LPS-induced NFκB subunits on simultaneous addition of peptide with LPS	LL-37	BMAP-27
p50	71 + 8	79 + 3
p65	75 + 4	73 + 0.9

%Inhibition of LPS-induced NFκB subunits on addition of peptide after LPS	LL-37	BMAP-27
p50	31 + 1.35	38 + 11
p65	35 + 4.2	47 + 8

Fig. 5. Cathelicidins LL-37 and BMAP-27 suppress LPS-induced translocation of NF-κB subunits p50 and p65. Nuclear extracts of THP-1 cells stimulated with LPS (10 ng/ml) in the absence or presence of LL-37 or BMAP-2 (5 μg/ml) and analyzed for NF-κB subunits p50 and p65 by ELISA. The *y*-axis represents relative light units (luminescence). Results shown are the mean ± 1 SD of three independent experiments (*, $P<0.05$; **, $P<0.01$). (A) Cells were stimulated with LPS in the absence or presence of the peptides (added simultaneously) for 60 min. (B) Cells were stimulated with LPS for 30 min, followed by the addition of the peptides for an additional 60 min.

T lymphocytes, neutrophils, and mast cells [46, 47]. Together, these data indicate that LL-37 has a series of overlapping functions, which promote chemotaxis of immune cells even in the presence of TLR agonists such as LPS.

Previous studies have suggested that the antiendotoxin activity of cationic peptides is in part a result of the direct binding of the cationic peptides to LPS and thereby, blocking the interaction of LPS with serum LBP [22]. Consistent with this, we demonstrated here that at low doses, human LL-37 and BMAP-27 largely inhibited LPS-mediated, proinflammatory responses as well as NF-κB migration into the nucleus, consistent with a major mechanism being direct binding to and neutralization of endotoxin. However, peptide interaction with LPS tends to be of relatively modest affinity (μM K_d), whereas the LBP-LPS interaction has a stable association constant, even at concentrations as low as 10 pg/ml LPS, and thereby, cannot be reversed easily [7], as demonstrated directly for antimicrobial peptides [22]. Similarly, the interaction of LPS with surface receptor MD-2 cannot be reversed easily. Furthermore, evidence has been presented that the suppression of LPS-induced, proinflammatory responses by endogenous cathelicidin LL-37 can involve a variety of mechanisms in addition to direct binding of the peptide to LPS [22, 36]. Thus, the ability of human LL-37 and BMAP-27 to retain their antiendotoxin activity, in part (≥60%), when added after 30 min of LPS stimulation, as well to retain their antiendotoxin activity when on delayed addition, even after removal of LPS from the extracellular media, indicates that the peptides have the potential to at least partially suppress LPS-induced, proinflammatory responses by mechanisms other than direct binding to LPS.

NF-κB is the key transcription factor that mediates LPS-induced responses by human and bovine cells [8, 9, 48]. Human LL-37 and BMAP-27 suppressed the LPS-induced nuclear translocation of NF-κB subunits p50 and p65 by more than 70% (Fig. 5). In addition, the ability of these peptides to inhibit LPS-induced NF-κB translocation was consistent with their effects at the transcriptional level, in suppressing LPS-induced gene expression of NFκB1 (encoding for p50) by ≥75% and RELA (NF-κB subunit p65) by ≥33% (Fig. 4; Table 2). Moreover, it was observed that LPS-induced gene expression of known NF-κB negative regulators, e.g., NFκBIL1 (in bovine cells) and NFκBIA (in human cells), was not neutralized completely in the presence of the peptides; indeed, BMAP-27 by itself induced the expression of NFκBIL1. The overall effect was a 90% decrease in the transcription of the TNF-α gene and ≥95% inhibition of LPS-induced TNF-α secretion in the presence of human and bovine peptides (Figs.

1 and 4). The ability of the cathelicidins to suppress LPS-induced nuclear translocation of NF-κB subunits was retained in part (30–40%) even on delayed addition of the peptides after 30 min of LPS stimulation, consistent with the suppression of LPS-induced secretion of TNF-α on delayed addition of the peptides in both mammalian species. Therefore, it can be concluded that the human and bovine cathelicidins suppress the LPS-induced translocation of NF-κB subunits influencing the overall inhibition of endotoxin-mediated, proinflammatory responses and that it appears that they do this in part by immunomodulatory mechanisms in addition to direct binding to LPS.

Although the overall effect of the human and bovine cathelicidin in suppressing LPS-induced, inflammatory responses was similar, it was interesting to note that the transcriptional responses of monocytic cells in presence of LPS alone and also the peptides alone differed. In general, the BMAP-27 appeared to induce up-regulation of several genes, e.g., NFKB1/p50, RELA, TNFAIP2, IL-10, MKP1/DUSP1, IκBκB, NFκBIL1, and TRAF2. In contrast, the response of monocytic cells to human LL-37 was modest. This may reflect differences in the responsiveness of human and bovine monocytic cells, although it should be noted that their antiendotoxin responses were highly similar. It is also possible that there is genuine heterogeneity in responses to different cationic host defense peptides, reflecting the microevolution of peptide function.

In conclusion, we demonstrate here that diverse cathelicidins of human and bovine origin, such as human LL-37 and BMAP-27, appear to have conserved, antiendotoxic functions. Thus, the immunomodulatory effects of mammalian cathelicidins may be conserved across species, a hypothesis that is consistent with the suggestion that they are functionally critical in maintaining homeostasis and limiting escalation of inflammation.

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