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Immunomodulatory activities of bovine host defense peptides (HDPs): context-dependent roles in inflammation and immune priming

Cristina Saubi[1], Kieran G. Meade[2,3,4,5], Sergi Travé-Asensio[1], Elena Garcia-Fruitós[1*] and Anna Aris[1*]

Abstract

Background Maintaining immune homeostasis is essential for livestock health and productivity, particularly in the face of infection or stress. Host defense peptides (HDPs), including β-defensins and cathelicidins, are key innate immune components with both antimicrobial and immunomodulatory properties. This study aimed to characterize the immunomodulatory effects of five bovine HDPs—BNBD1, BNBD3, LAP, Bac5, and BMAP27—on peripheral blood mononuclear cells (PBMCs) and bovine turbinate (BT) epithelial cells, under both basal and lipopolysaccharide (LPS)-stimulated inflammatory conditions. Cytokine secretion, cell viability, and real-time epithelial cell behavior were assessed to evaluate peptide-specific immune modulation.

Results Under non-stimulated conditions, LAP stimulated the secretion of IL-8 (LAP $p < 0.0001$). In LPS-stimulated PBMCs, prophylactic HDP treatment (4 h pre-LPS) amplified inflammatory cytokine secretion. BNBD3 and LAP significantly increased IL-1β, IL-6, TNF-α, IFN-γ ($p < 0.05$), and chemokines such as IL-8 and MIP-1α ($p < 0.01$), suggesting an immune-priming effect that may enhance responsiveness to subsequent LPS stimulation. Notably, BNBD3 and LAP failed to induce a comparable inflammation when added 0.5 h after LPS, highlighting the context-dependent nature of HDP action. Conversely, BMAP27 demonstrated anti-inflammatory activity by reducing LPS-induced IL-1β, IFN-γ, and IL-10 ($p < 0.001$), irrespective of timing. Bac5 increased IL-8 secretion ($p < 0.001$) and MCP-1 ($p < 0.05$), suggesting a chemotactic effect. Cell viability assays confirmed that none of the peptides exhibited cytotoxicity in PBMCs at tested concentrations, although BMAP27 reduced lymphocyte numbers (40% decrease, $p < 0.0001$), possibly indicating selective immunoregulatory effects. In BT cells, Bac5 enhanced proliferation (11% increase, $p < 0.05$), while BNBD1 (23% decrease, $p < 0.01$) and BMAP27 (10% decrease, $p < 0.05$) mildly reduced cellular impedance, reflecting divergent impacts on epithelial dynamics.

Conclusions These findings reveal distinct immunomodulatory profiles among bovine HDPs, ranging from pro-inflammatory (BNBD3, LAP) to suppressive (BMAP27), and underscore the importance of treatment timing. The immune-priming capacity of certain HDPs suggests potential use as prophylactic agents to enhance resilience to infections, while suppressive peptides like BMAP27 may serve therapeutic roles in resolving excessive inflammation.

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*Correspondence:
Elena Garcia-Fruitós
elena.garcia@irta.cat
Anna Aris
anna.aris@irta.cat

Full list of author information is available at the end of the article

Importantly, the variability observed among individual animals emphasizes the need for personalized approaches in immunomodulation. Overall, this study provides novel insights into the immunological functions of bovine HDPs, supporting their potential as alternatives or adjunctive therapies to antibiotics in veterinary medicine.

Keywords Host defense peptides (HDPs), Immunomodulation, Cytokine secretion, Bovine immunity, Antibiotic alternatives

Background

Immune responses in livestock exhibit continual fluctuations after establishment *in utero* to functional maturation in the neonate and throughout the lifetime of the individual as a result of commensal colonisation and pathogen challenge. Maintaining a balanced immune response in the face of such prokaryotic diversity and opportunism is essential for the long-term health, welfare, and productivity of livestock [1, 2]. Ideally, a prompt and effective innate immune response will eliminate pathogens without the need for adaptive immune responses, but excessive or sustained activation of innate immunity can result in pathological inflammation and tissue damage [3–5]. Compromised cellular integrity can contribute to pathological inflammation, and then, commensal bacteria such as *Escherichia coli*, a common Gram-negative bacterium in the gastrointestinal tract that usually supports gut health, can trigger disease [6, 7]. When immune responses are unbalanced, severe pathology leading to organ dysfunctions and increased susceptibility to secondary infections can result, as observed in sepsis [3, 4, 8–10], bovine respiratory disease (BRD) [11, 12], and Covid-19 [13, 14].

Molecules involved in maintaining an optimal immune balance between protection and pathology include Host Defense Peptides (HDPs). HDPs are key effector molecules of the innate immune system with pleiotropic activities, including direct antimicrobial activity against bacteria, viruses, fungi, and even cancer cells [15, 16]. Beyond their widely studied antimicrobial roles, HDPs also exhibit immunomodulatory functions at lower concentrations, which are thought to be more relevant in physiologic conditions [16–19].

In the context of maintaining protective epithelial barriers, human cathelicidin LL-37 and human β-defensin 3 (hBD3) accelerate wound healing by promoting fibroblast migration and proliferation; enhancing macrophage and neutrophil infiltration into injured tissues to resolve inflammation; and inducing the expression of enzymes involved in angiogenesis and extracellular matrix remodeling [20, 21].

In response to infection, HDPs recruit immune effector cells through both direct and indirect chemotactic mechanisms. The LL-37, hBD3, and human β-defensins 2 (hBD2) bind to chemokine receptors, attracting neutrophils, monocytes, eosinophils, T cells, and immature dendritic cells to infection sites [22–30]. They also induce the secretion of chemokines such as IL-8 to attract neutrophils [31, 32] and MIP-1β/CCL4 [33, 34] to recruit monocytes, dendritic and T cells [35]. As the response progresses, HDPs modulate inflammation by regulating cytokine levels. hBD3 induces pro-inflammatory cytokines IL-1β and IL-6 in monocytes [36] and IL-6, TNF-α, and IL-1α in macrophages [37]; while the cathelicidin Bovine Myeloid Antimicrobial Peptide 28 (BMAP28) enhances IL-1β without affecting IL-6 in macrophages [38]. Conversely, LL-37 and hBD2 upregulate anti-inflammatory cytokines IL-10 and IL-1RA [39–42]; and LL-37 and hBD3 suppress LPS-induced IL-6, IL-1β, and TNF-α [39, 43–46].

HDPs also influence cell effector functions. In neutrophils, LL-37 regulates reactive oxygen species (ROS) production [47, 48], promotes neutrophil extracellular trap (NET) formation [49, 50], and, along with hBD3, inhibits apoptosis to prolong immune activity [51, 52]. Moreover, they facilitate the transition to adaptive immunity. LL-37 enhances antigen uptake [53] and promotes antigen-presenting cell (APCs) maturation [54–56], bridging innate and adaptive immunity. Moreover, it further guides T_H cells toward T_H 1 or T_H 2 responses [53].

To date, most of these immunomodulatory activities have been characterized in detail for only a limited number of human HDPs, most notably the cathelicidin LL-37 and certain β-defensins. In contrast, much less is known about the regulatory functions of bovine HDPs, since most studies have focused on expression patterns and antimicrobial functions. For instance, the cathelicidin BMAP28 and the β-defensin Lingual Antimicrobial Peptide (LAP) are constitutively expressed in the mammary gland to protect from infection [57, 58], whereas the secretion of β-defensin Tracheal Antimicrobial Peptide (TAP), and also LAP, is induced in response to infection stimuli like LPS [59–62]. In addition, decreased levels of HDPs have been associated with infection susceptibility. TAP-induced expression, for example, is reduced by stress (glucocorticoids) and Bovine Virus Diarrhea Virus (BVDV), factors related to a higher risk of infections [63]. These findings underscore the importance of HDPs in bovine immunity, yet their functional roles in immune regulation remain largely uncharacterized.

In this study, we investigated the immunomodulatory effects of five bovine HDPs on peripheral blood mononuclear cells (PBMCs) and bovine turbinate (BT) epithelial cells. The selected HDPs included β-defensin – bovine neutrophil β-defensins 1 (BNBD1), bovine neutrophil β-defensin 3 (BNBD3), and the lingual antimicrobial peptide (LAP) – as well as cathelicidins – bactenecin 5 (Bac5) and bovine myeloid antimicrobial peptide 27 (BMAP27). Their effects were assessed by quantifying cytokine and chemokine secretion in PBMCs under basal conditions and before and after LPS stimulation, also evaluating their cytotoxicity on PBMCs and BT cells. The present work aimed to characterize the immunomodulatory potential of these peptides and identify differences in their activity that could guide strategies to enhance disease resilience and therapeutic interventions in cattle.

Materials and methods

Synthetic peptides

Mature sequences of bovine neutrophil β-defensin 1 (BNBD-1, UniProt_P46159) and β-defensin 3 (BNBD-3, UniProt_P46161), as well as the Lingual Antimicrobial Peptide (LAP, UniProt_Q28880) and cathelicidins 2 (Bac5, UniProt_P19660) and 6 (BMAP27, UniProt_P54228) were chemically synthesized in the linear form (Table 1) by RoyoBiotech (Shanghai, China). Peptides were resuspended in 20 mM HEPES (pH = 5.2) prepared with endotoxin-free water, aliquoted, and stored at – 80 °C or re-lyophilized for longer storage periods.

Animals and blood sampling

Blood was collected from cattle aged 23–24 months old, owned by IRTA experimental farm, in EDTA-K2 blood collection tubes (367861, BD Vacutainer, Eysins, Switzerland) following a protocol approved by the Animal Care Committee of the Generalitat de Catalunya (project number 12287).

PBMC isolation

PBMCs were isolated from fresh blood either using pooled blood from multiple animals (13 and 21 animals) or individual samples obtained from three healthy female Holstein cows. Blood was diluted twofold in Dulbecco's Phosphate Buffered Saline (D-PBS, 14190-094, Gibco) and transferred to a PBMC isolation tube (SepMate™ – 50 mL, StemCell Technologies) previously loaded with 15 mL of Histopaque 1077 (10771, Sigma-Aldrich). Tubes were centrifuged at 1,500 × g for 25 min at room temperature (RT) and without brake. We collected the buffy coat and performed three washes with D-PBS, centrifuging at 300 × g for 10 min at RT with low braking force. Cells were resuspended in RPMI Medium 1640 + GlutaMAX$^{\text{TM}}$-I (61870-010, Gibco), supplemented with 10% low-endotoxin Foetal Bovine Serum (FBS MaxSpec, A4766801, Thermo Fisher) and 1% Penicillin/Streptomycin (10,000 U/mL, 15140-122, Gibco) and counted using the auto-haematology analyzer (BC-5150, Mindray).

Table 1 Sequence of selected HDPs

Name	Sequence
BNBD1	DFASCHTNGGICLPNRCPGHMIQIGICFRPRVKCCRSW
BNBD3	QGVRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW
LAP	VRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK
Bac5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP
BMAP27	GRFKRFRKKFKKLFKKLSPVIPLLHLG

PBMC stimulation

In 24-well plates (TC-plate 24w, 83.3922, Sarstedt), 400 µL of PBMC suspension was added to each well, ensuring a final count of 5×10^5 cells/well. Plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 overnight to recover cells from the stress of the isolation process and improve viability. PBMCs were treated with synthetic peptides (50 µL) at concentrations of 1 or 4 µM for 4 h before an immune challenge with 100 ng/mL (50 µL) of *Escherichia coli* O111:B4 lipopolysaccharides (LPS, L4391, Merck) or 0.5 h after LPS treatment. Controls of cells grown with 50 µL of protein buffer and stimulated with LPS without any HDP were included. PBMCs were also incubated with HDP treatments alone to test if HDPs stimulated the secretion of cytokines. Cells were then incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. On the one hand, supernatants were first centrifuged at 600 × g for 5 min at 4 °C to remove cells in suspension, followed by a second centrifugation at 10,000 × g for 2 min at 4 °C to eliminate cell debris. Supernatants were kept at – 80 °C until use. On the other hand, cells were recovered and resuspended in a final volume of 100 µL for cell counting with the auto-haematology analyzer (BC-5150, Mindray).

Cytokine measurement

IL-1β and IL-6 were quantified using commercial ELISA kits for bovine IL-1β (ESS0027, ThermoFisher) and IL-6 (ESS0029, ThermoFisher) and following the manufacturer's protocol. IL-8 was quantified following the protocol previously described [64] using 5% Tween 20 in D-PBS as a blocking buffer. MILLIPLEX multiplex assay for bovine cytokines/chemokines (Millipore) was performed using Luminex technology (MAGPIX automated analyzer, Luminex) to measure: IL-8, IL-1β, TNF-α, IL-17 A, IL-6, IFN-γ, IL-10, MCP-1, MIP-1α, and TGF-β1.

HDP cytotoxicity in PBMCs

HDP toxicity on PBMCs was evaluated using MTT assays. Briefly, 80 µL of PBMC suspension was added to each well of 96-well plates (96w Nucleon Delta-Treated, 167008, ThermoFisher Scientific), ensuring a final count of 1×10^5 cells/well with 10 µL extra of cell media. The cell number per well was adjusted to match the PBMC experiments done in 24-well plates, ensuring a consistent final cell concentration of 1×10^6 cells/mL in all experiments.

Cells were rested overnight and then treated with 10 µL of HDPs at a final concentration of 1 and 4 µM for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell cytotoxicity was assessed using an MTT kit (Cell Proliferation Kit I – MTT, 11465007001, Roche) and following the manufacturer's protocol. A low cytotoxicity control (LC) with protein buffer and a high cytotoxicity control (HC) with Triton X-100 were included. For this, cells were treated with 10 µL of Triton^{TM}X-100 (T8787, Sigma-Aldrich) diluted 1:10 in water for 15 min at 37 °C in a humidified atmosphere with 5% CO_2 before adding the MTT reagent solution. Complete solubilization of the purple formazan crystals was confirmed, and absorbance was measured at 570 nm with a reference wavelength of 650 nm using a Lumistar plate reader (LUMIstar® Omega, BMG Labtech).

Cell viability was calculated as:

Equation 1 $\text{Cell viability } (\%) = \frac{(\text{sample}-\text{HC})}{(\text{LC}-\text{HC})} \times 100.$

Real-time cell analysis (RTCA) of BT cells

Bovine Turbinate cells (BT CRL-1390, ATCC) were grown in DMEM media with 4.5 g/L glucose, L-glutamine and sodium pyruvate (10–013-CV, Corning) supplemented with 10% FBS (10082-147, Gibco) and 1% penicillin/streptomycin (5,000 U/mL, 15070063, Gibco) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were detached with 0.25% trypsin-EDTA (25200056, Gibco) for subcultivation. Real-time cell growth was analyzed using a xCELLigence RTCA DP instrument (Agilent Technologies) with 16-well E-plates (5469830001, Agilent Technologies). Wells were filled with 100 µL/well of cell culture media and left to equilibrate at RT for 30 min before inserting the plate in the analyzer for background measurement. A volume of 90 µL BT cells was plated on each well to obtain 1×10^4 cells/well and left at RT for 30 min for the cells to settle. Cell adhesion and growth were tracked by measuring the impedance every 15 min until cells reached the growing phase (~ 24 h). Cells were treated with 5 µL/well of synthetic peptides at 4 µM and/or LPS at 100 ng/mL and incubated for 24 h while measuring impedance every 15 min. All samples were run in duplicates and controls with protein buffer and LPS alone were included. Results were analyzed using the RTCA software Pro. Cell index (CI) is the unitless parameter used to evaluate the impedance of electron flow caused by adherent cells on the golden chip, which enables tracking cell activity, including adherence, proliferation, and spreading.

Data and statistical analysis

Outliers were detected using the Interquartile Range (IQR) method, where values falling outside 1.5 times the IQR below the first quartile or above the third quartile were excluded. Normality was assessed using Shapiro-Wilk and Anderson-Darling tests and log transformation was applied to datasets that did not meet normal distribution to improve normality. Statistical analyses were performed using a mixed-model ANOVA followed by least square means (LSMeans) tests, considering main effects and interactions among treatment and concentration, as well as time of administration relative to LPS addition. The animal effect was included in the model when blood from individual animals was used. Dunnett's post-hoc test was applied to compare treatments to the control. One-way or Two-way ANOVA were applied for cytotoxicity assays, as appropriate. Statistical analyses were performed in JMP Pro software v17.0.0, and figures were created using GraphPad Prism v8.3.0.

Results

The effects of HDPs on PBMC cytokine secretion

The immunomodulatory activity of five bovine HDPs was first explored using pooled PBMCs from multiple animals. Only BNBD1 significantly increased IL-6 secretion compared to the untreated control ($p < 0.05$), Fig. 1B). The secretion of the chemokine IL-8 increased by BNBD1, Bac5, and BMAP27, with BNBD1 achieving the greatest effect ($p < 0.01$, Fig. 1C). Additionally, BNBD1, BNBD3, and LAP promoted IL-1β secretion, with BNBD1 also producing the highest response ($p < 0.05$, Fig. 1D).

Experiments were repeated using PBMCs from three individual animals and measuring cytokines IL-8 and IL-1β. IL-8 secretion was elevated by all treatments except BNBD1, with LAP inducing the highest levels ($p < 0.0001$, Fig. 2B). A modest secretion of IL-1β was detected after BMAP27 treatment ($p < 0.01$, Fig. 2C). No significant interaction between HDP treatment and concentration was observed in any analysis.

Preventive roles of HDPs during inflammation

To assess the prophylactic potential of HDPs, PBMCs were pretreated with peptides 4 h before LPS stimulation. No changes were observed in the IL-6 secretion upon HDP treatments. Bac5 was the only one to increase IL-8 regardless of the concentration used ($p < 0.01$, Fig. 3C). There was a significant interaction between treatments and concentration for IL-1β analyses ($p < 0.05$, Fig. 3D). All HDPs at 4 µM enhanced IL-1β secretion, except BMAP27, which suppressed LPS-induced IL-1β ($p < 0.0001$).

Experiments with PBMCs from individual animals confirmed that BNBD3 and LAP pretreatment boosted the LPS-induced secretion of proinflammatory IL-1β ($p < 0.05$, Fig. 4C) and the chemokine IL-8 ($p < 0.01$, Fig. 4B); with Bac5 also enhancing IL-8 ($p < 0.05$, Fig. 4B). Meanwhile, BMAP27 significantly decreased IL-1β secretion induced by LPS ($p < 0.001$).

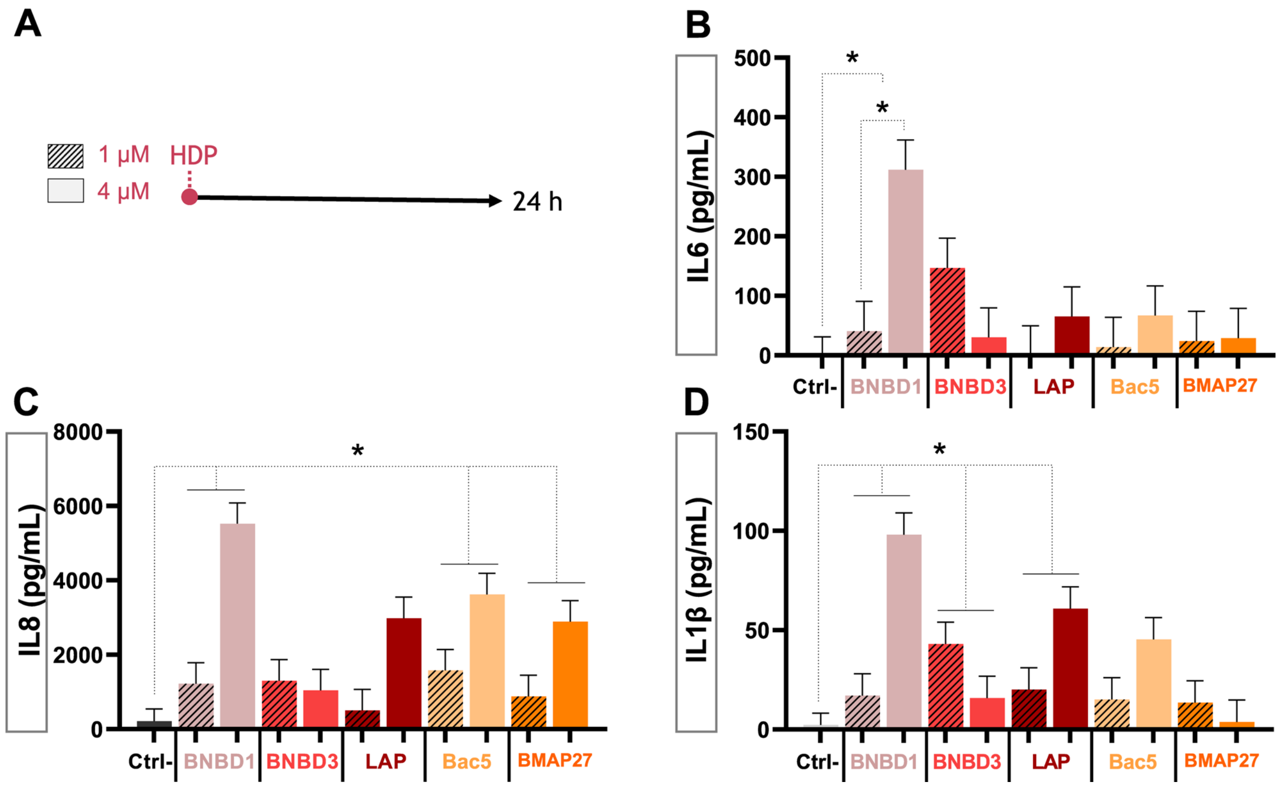

Fig. 1 Cytokine response of bovine PBMCs to HDPs. Mononuclear cells were isolated from pooled bovine peripheral blood and treated with either 4 or 1 µM of synthetic HDPs for 24 h (**A**). Cell culture supernatants were collected to quantify the secretion of IL-6 (**B**), IL-8 (**C**), and IL-1β (**D**) by ELISA. Statistical analysis was performed using the Least Squares Means (LSMeans) method. The model included simple effects and a two-way interaction between treatment and concentration; however, the interaction was not significant, so only simple effects are shown. Dunnett's post hoc test was used to compare each group with the negative control. The experiment was repeated twice, each run performed in duplicate, resulting in four independent data points (n=4). Data are presented as the model-adjusted means ± SEM from four replicates. Statistical significance is denoted by (*): $p \le 0.05$. Horizontal lines above different bars indicate no significant differences between those groups, whereas dashed lines indicate statistical differences

BNBD3 and LAP also increased other proinflammatory cytokines such as TNF-α, IL-17 A, and IL-6, and IFN-γ, anti-inflammatory cytokine IL-10, along with chemokine MIP-1α (Fig. 5). MCP-1 was increased by BNBD3 pretreatment ($p < 0.05$, Fig. 5H) but not by LAP. On the contrary, BMAP27 consolidated its anti-inflammatory role –previously observed with pooled PBMCs– by reducing IL-17 A and IFN-γ ($p < 0.0001$, Fig. 5C and E), but also the anti-inflammatory cytokine IL-10 ($p < 0.0001$, Fig. 5F). BNBD1 showed minor effects, increasing IL-17A and IL-10 ($p < 0.001$, Fig. 5C and F). Treatment with any of the HDPs did not alter TGF-β1 cytokine expression.

Therapeutic roles of HDPs during inflammation

When HDPs were administered 0.5 h after LPS stimulation, BMAP27 and Bac5 increased IL-8 secretion in pooled PBMCs ($p < 0.0001$, Fig. 3C), but this was not replicated in PBMCs from individual animals (Fig. 4B). Remarkably, BNBD3 and LAP administered after the LPS insult did not elicit proinflammatory responses observed under prophylactic simulations (Fig. 5). BMAP27 consistently reduced IL-1β, IL-17 A, IFN-γ, and IL-10 levels (Figs. 3, 4 and 5), demonstrating a suppressive effect independent of concentration (1 or 4 µM) and administration timing (before or after LPS).

Statistical analysis revealed interaction effects between treatment and the time of LPS administration (preventive or therapeutic) for IL-8 ($p < 0.001$). For IL-1β, the three-way interaction (treatment × concentration × time) was significant when pooled blood was used ($p < 0.05$), but not when blood from individual animals was analyzed ($p = 0.09071$). In the latter case, the two-way interaction treatment × concentration was significant ($p < 0.01$).

The effect of HDPs on PBMCs and BT cell growth and viability

HDPs did not impair the metabolic activity of PBMCs measured by MTT (Fig. 6A) but increased the cell activity after 4 µM BMAP27 treatment. In contrast, the same treatment caused a significant reduction in lymphocyte count (Fig. 6B). The possible effect of HDPs on epithelial cells was assessed by Real-time Cell Analysis on BT cells using an xCELLigence RTCA DP instrument, whereas secretion of IL-6, IL-8, and IL-1β was not quantifiable

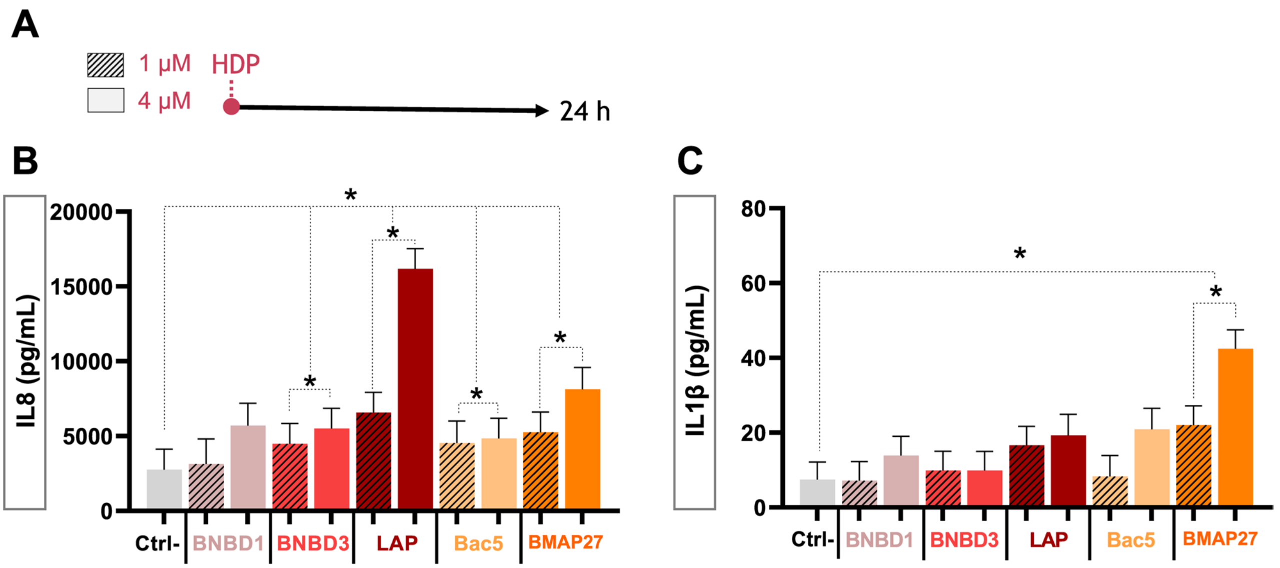

Fig. 2 Effect of HDPs on IL-8 and IL-1β secretion across individual animals. The experiment was repeated with blood from three different animals, each of which was used in a separate parallel experiment to account for individual variability. HDPs were added and supernatants collected after 24 h (**A**) to quantify IL-8 (**B**) and IL-1β (**C**). Statistical analysis followed the same model as in the previous figure, but included individual animal variability as a random effect. The interaction between treatment and concentration was not significant, so only simple effects are shown. The experiment was repeated three times using different animals, each run performed in duplicate, resulting in six independent data points (*n*=6). Data are presented as the model-adjusted means ± SEM, and statistical significance is denoted by (*): $p \leq 0.05$. Dashed lines indicate statistical differences between specific groups

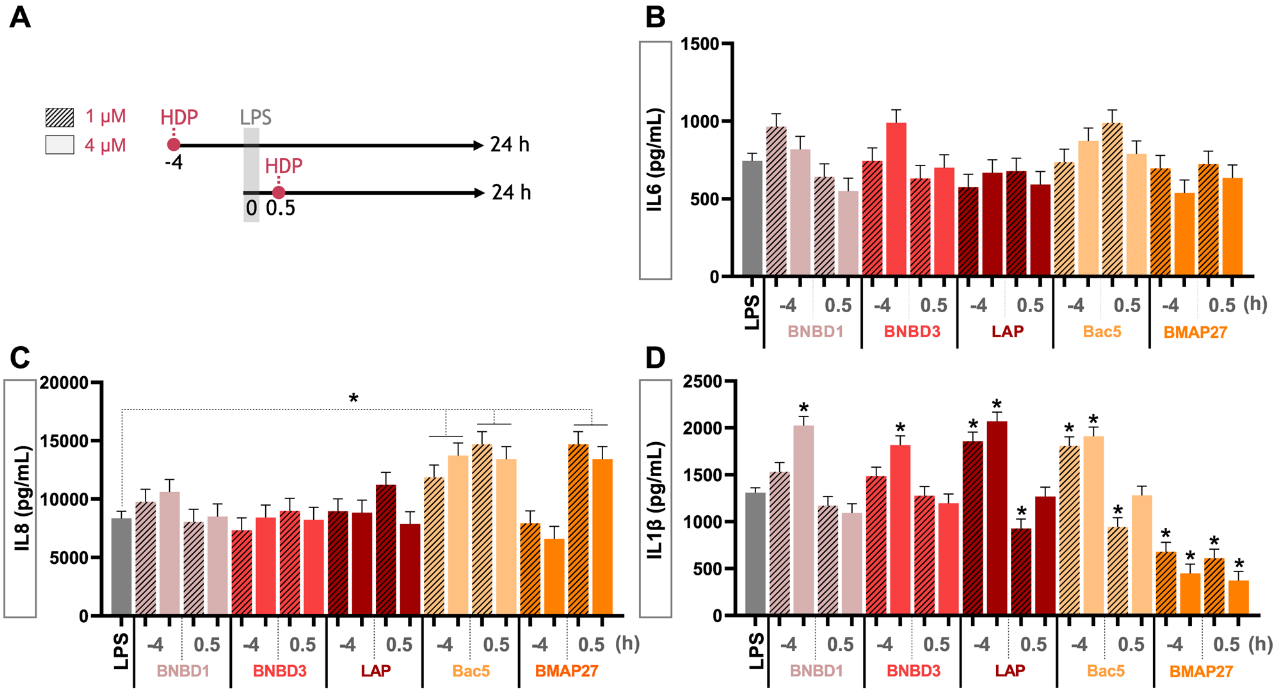

Fig. 3 Cytokine response of pooled bovine PBMCs to HDPs given an LPS challenge. PBMCs from pooled bovine peripheral blood were treated with either 4 or 1 µM of synthetic HDPs, together with an immune challenge using *Escherichia coli* O111:B4 lipopolysaccharide (LPS, 100 ng/mL). HDPs were added 4 h before (−4 h) or 30 minutes (0.5 h) after the LPS challenge (**A**). After 24 h of incubation, cell culture supernatants were collected to quantify the secretion of IL-6 (**B**), IL-8 (**C**), and IL-1β (**D**) by ELISA. Statistical analysis was performed using the LSMeans method. The model included simple effects, two-way, and three-way interactions (treatment × concentration × time). For IL-8, treatment × time was significant ($p < 0.001$) and shown. For IL-1β, the triple interaction was significant ($p < 0.05$) and shown. Dunnett's post hoc test was used to compare each group with the LPS control. Each condition was tested once in duplicate. Data are presented as the model-adjusted means ± SEM. Statistical significance is denoted by (*): $p \leq 0.05$. Horizontal lines above different bars indicate no significant differences between those groups, whereas dashed lines indicate statistical differences

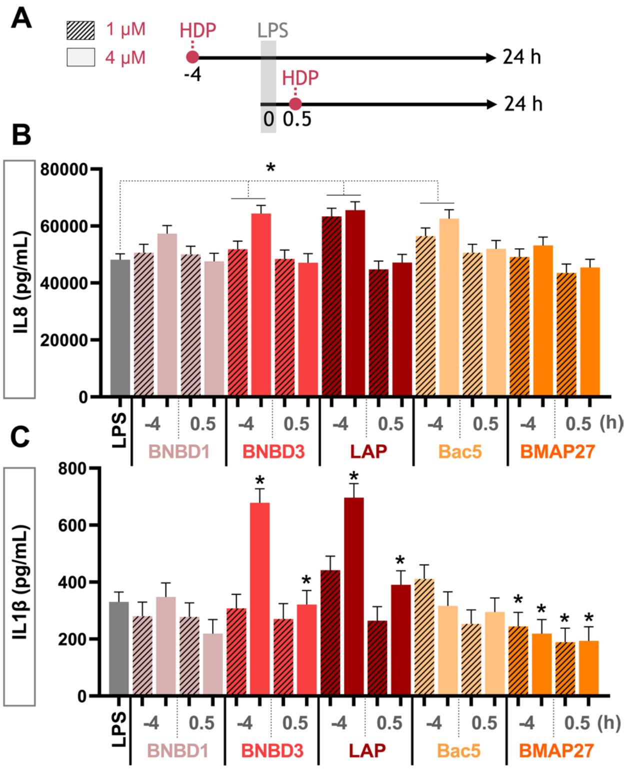

Fig. 4 Effect of HDPs on IL-8 and IL-1β secretion given an LPS immune challenge across individual animals. HDPs were added either 4 h before (−4 h) or 30 min after (0.5 h) LPS (**A**). IL-8 (**B**) and IL-1β (**C**) were quantified. Statistical analysis was performed using the LSMeans method. For IL-8, the three-way interaction was not significant; treatment × time was significant ($p < 0.001$) and shown. For IL-1β, the three-way interaction was not significant; treatment × concentration was significant ($p < 0.01$) and shown. The experiment was repeated three times using different animals, each run performed in duplicate (n=6). Data are presented as the model-adjusted means ± SEM, and statistical significance is denoted by (*): $p \le 0.05$. Horizontal lines above different bars indicate no significant differences between those groups, whereas dashed lines indicate statistical differences

using ELISA techniques (Results not shown). Cells were treated with HDPs at 4 μM or LPS 100 ng/mL, and cell behaviour was compared to cells alone for 24 h. After 10 h of incubation, significant reductions in the cell index (CI) were observed with both LPS (8% reduction, $p < 0.05$) and BNBD1 (23% reduction, $p < 0.01$). These effects persisted throughout the experiment, with LPS reducing CI to 25% ($p < 0.0001$), surpassing BNBD1. By 24 h, BMAP27 treatment induced a slight but significant decrease in the cell index (10%, $p < 0.05$), whereas Bac5 caused a significant increase (11%, $p < 0.05$) compared to the negative control.

Discussion

In light of growing issues with antimicrobial resistance (AMR), there is an urgent need to reduce our collective dependence on exogenous antimicrobials in agriculture, specifically antibiotics and anthelmintics [65–68]. Host Defence Peptides (HDPs) offer potential new therapeutics, but their immunological mechanisms of action have not been extensively investigated in livestock species. While previous studies have explored the antimicrobial activity of bovine HDPs, their immunomodulatory role on cytokine secretion in PBMCs is not well characterized. This study provides an initial comparative analysis of the immunomodulatory profiles of five bovine HDPs—BNBD1, BNBD3, LAP, Bac5, and BMAP27—representing the two main HDP subfamilies: β-defensins and cathelicidins. Preferentially expressed at mucosal surfaces, these peptides can influence both local and systemic immune responses.

Results showed that HDPs had a limited impact on PBMCs in the absence of LPS stimulation. Main changes were observed in IL-8 secretion, which was increased consistently by most of HDPs (Figs. 1 and 2). In contrast, cytokine and chemokine modulation by HDPs became more pronounced in the presence of LPS (Figs. 3, 4 and 5). Under these conditions, BNBD3 and LAP consistently promoted a proinflammatory and chemotactic response, significantly increasing IL-1β, IL-17 A, IL-6, TNF-α, IFN-γ, and chemokines IL-8 and MIP-1α (Fig. 5). BNBD3 also stimulated the chemokine MCP-1 and the anti-inflammatory IL-10, suggesting a dual role: initiating immune activation while simultaneously engaging regulatory mechanisms to prevent overactivation. BNBD1 induced a narrower yet similarly balanced cytokine response, marked by the secretion of IL-17 A and IL-10 (Fig. 5). Although specific studies on BNBD1, BNBD3, and LAP are lacking, human β-defensin 3 (hBD3) has been shown to promote secretion of pro-inflammatory IL-1β and IL-6 [36], and human β-defensin 2 (hBD2) to induce LPS-stimulated IL-10 [42].

Amongst cathelicidins, BMAP27 demonstrated a predominantly immune-dampening function, reducing LPS-induced IL-1β (Figs. 3 and 4) and Il17A, IFN-γ, and IL-10 (Fig. 5). These results align with previous studies reporting that BMAP27 does not stimulate IL-8 secretion in monocytic cells, but does suppress LPS-induced signalling, including the expression of IL-10 [40]. That same study also reported reductions in IL-8 and TNF-α following LPS stimulation, which were not observed. These discrepancies could be due to methodological differences, including variations in cell type, HDP and LPS concentrations, and the use of gene expression analysis instead of cytokine quantification [40]. In contrast, Bac5 enhanced IL-8, IL-6 and MCP-1 production in response to LPS (Figs. 4 and 5), indicating a role in immune cell recruitment. Overall, these findings support the concept of HDPs as pleiotropic molecules with overlapping yet distinct immunological roles.

MTT assay results showed that none of the peptides were cytotoxic to PBMCs at 4 μM. Instead, BMAP27 increased metabolic activity, while independent cell

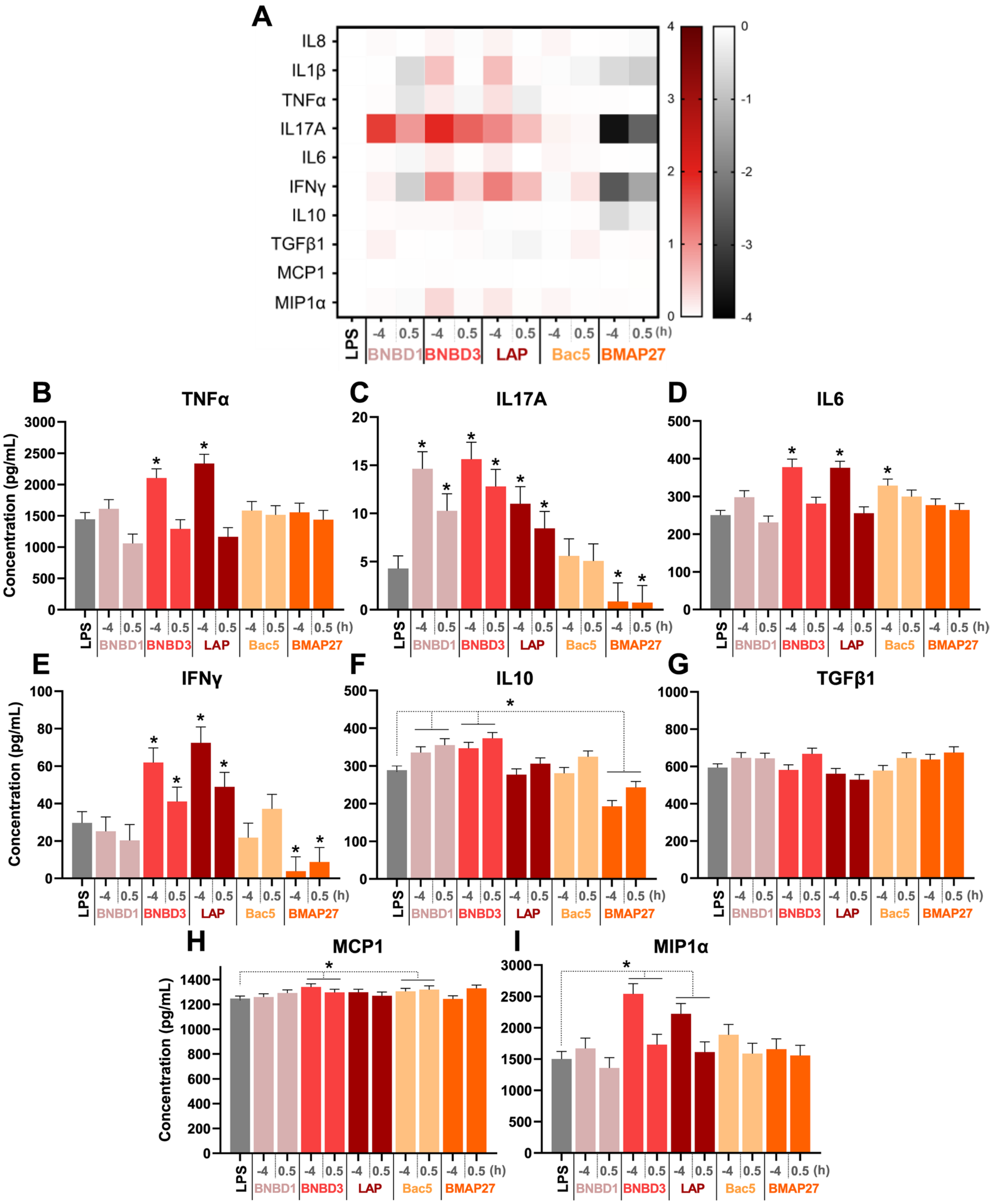

Fig. 5 Modulation of cytokine and chemokine secretion by HDPs in LPS-stimulated bovine PBMCs isolated from individual animals. A broader cytokine and chemokine panel was analysed following 4 µM HDP treatment at −4 h and 0.5 h relative to LPS. (**A**) Heat-map representing log2 fold changes in cytokine levels compared to LPS alone. IL-8 and IL-1β were calculated from ELISA results (Figure 3). Remaining analytes were quantified via multiplex immunoassay. (**B** – **I**) Effects of HDPs on individual cytokine concentrations. Statistical analysis was performed using the LSMeans method accounting animal variability. The interaction between treatment and time of administration was significant for IFN-γ, IL-17A, TNF-α, and IL-6, and is shown. This interaction was not significant for IL-10, MCP-1, and MIP-1α; therefore, the protein effect is represented. The experiment was repeated three times using different animals, each run performed in duplicate (*n*=6). Data are presented as model-adjusted means ± SEM, and statistical significance is indicated by (*): $p \leq 0.05$. Solid lines denote no significant differences, dashed lines indicate statistical differences

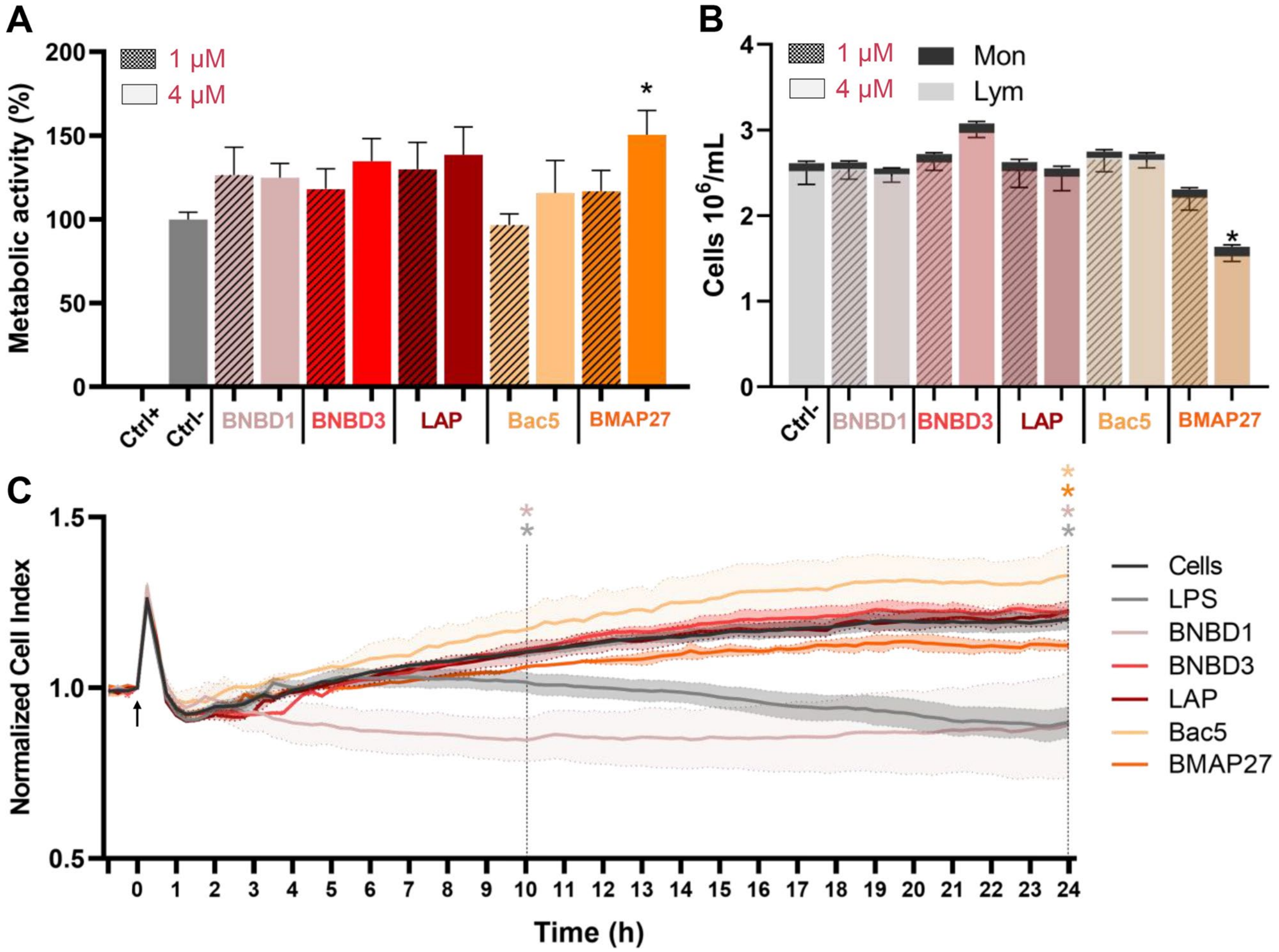

Fig. 6 Cytotoxicity of HDPs on PBMCs from individual animals and bovine turbinate (BT) cells. (**A**) The metabolic activity of PBMCs was measured using the MTT assay. Data are represented relative to the negative control (cells treated with protein buffer only). (**B**) After a 24-h incubation of cells with HDPs, cells were recovered and concentrated in a controlled volume. Cell counting was performed using an auto-haematology analyzer. Data represented as the mean ± SEM. One-way ANOVA with Dunnett's post-hoc was used to calculate significant differences compared to the negative control (Ctrl -). Statistical significance is represented by (*): $p \leq 0.05$. (**C**) Real-time cell analysis of BT cells treated with BNBD1, BNBD3, LAP, Bac5, and BMAP27 at 4 μM or LPS (100 ng/mL). Cells were grown in T-flasks and then seeded onto e-plates at 10,000 cells/well. HDPs or LPS were added after cells were accommodated and in the growth phase (t = 0 h). A control with cells alone was included. Mean values are represented as solid lines and SEM as dotted. Statistical differences compared to the control were calculated using a 2-way ANOVA and are represented by (*): $p \leq 0.05$ at t=10 h and 24 h

counts revealed a reduction in lymphocyte numbers (Fig. 6). Previous studies have reported that BMAP27 is non-cytotoxic to bovine epithelial cells [69] or when injected intraperitoneally in mice [70], but it has been suggested to possess anti-cancer [71–73] and pro-apoptotic activities [71, 73]. Risso et al.. demonstrated that BMAP27 at 3 μM induces membrane permeabilization and programmed cell death in activated, non-cancerous lymphocytes [73]. This could potentially be due to its preferential targeting of membranes exposing phosphatidylserine (PS), as observed by Yang et al. [72]. Under homeostatic conditions, PS is located in the inner leaflet of the plasma membrane, but externalized during apoptosis and cell stress – even in response to cytokines like IFN-γ [74] – serving as a signal for phagocytosis and immune suppression [75–77]. Apoptotic cell death promotes the release of anti-inflammatory cytokines and the inhibition of pro-inflammatory molecules and TLR signaling [75]. Consistent with this, BMAP27 was shown to induce IL-10 secretion in PBMCs [40], further supporting its immunomodulatory potential. Thus, BMAP27 may help prevent excessive immune activation, hyperinflammation, and collateral tissue damage by selectively inducing apoptosis in activated or stressed lymphocytes while promoting anti-inflammatory responses.

Real-time cell analysis (RTCA) showed that BNBD1 impaired cell growth, as the cell index remained stable throughout the experiment and, at 24 h, was comparable to that of LPS-treated cells. The fact that its effect is analogous to that of LPS suggests a potential involvement of the inflammatory response pathway, which may be linked to growth arrest. Nevertheless, future work is required to elucidate this aspect, as we were unable to detect cytokine secretion from the BT cells in our experimental conditions. On the other hand, there was a slight increase in cell index with the treatment of Bac5 compared to the control, suggesting that Bac5 can promote cell proliferation (Fig. 6). These findings are consistent with those reported by Tomasinsig et al., who observed that Bac5 promoted fibroblast proliferation at concentrations above 10 μM [78]. Since epithelial cell proliferation is key in wound healing, Bac5 could be involved in tissue repair. This aligns with the known roles of HDPs in wound healing through a multifaceted array of mechanisms, including modulation of cytokine and chemokine secretion, and stimulation of cell proliferation and migration [79, 80]. For instance, LL-37 has been demonstrated to induce epithelial cell proliferation and migration, helping in the re-epithelialization of skin wounds [81, 82].

Interestingly, the timing of HDP administration significantly influenced peptides' activity. Overall, HDP pretreatment 4 h before the LPS insult (−4 h) induced greater effects on cytokine production than when administered after (0.5 h) (Figs. 3, 4 and 5). This was evident with LAP, BNBD3, BNBD1, and Bac5, which promoted secretion of proinflammatory cytokines IL-8, IL-6, and TNF-α only when given before the immune challenge (Fig. 5). In contrast, HDP administration after the LPS challenge (0.5 h) resulted in a more restrained cytokine response. These findings suggest that HDPs may prime the innate immune system for enhanced responsiveness to future pathogen encounters.

This priming effect aligns with the concept of trained immunity, in which innate cells develop an enhanced ability to respond to secondary challenges, often through increased secretion of IL-1β, TNF-α, and IL-6 [83, 84]. However, elevated or sustained levels of these cytokines have been associated with worsened infection-related pathology [85–88]. Thus, the tempered response following post-LPS treatment suggests that these peptides may prime innate immunity while also preventing harmful hyperinflammation.

Using a PBMC pool in a preliminary experiment is an efficient strategy to reduce variability, save resources, and obtain a robust signal to guide the design of definitive experiments with individual donors. It is crucial to understand that using pools is a tool for preliminary phases, but it does not replace individual analysis in the definitive study. This provides a result that is more representative of an average immune response or the study population, rather than a result that might be an outlier from a single donor. As expected, differences were detected between experiments using PBMCs from pooled blood or individual animals. While pooled samples provided a general overview, individual variability was evident and impacted on results. Previous studies have reported significant inter-individual variation in cytokine profiles in basal levels and LPS-induced responses, which could be linked to disease susceptibility [5]. Therefore, a larger number of animals would be necessary to account for this variability and strengthen the findings. However, herein, the first use of pooled samples allowed us to select the most functional concentrations and incubation times.

While this study provides novel insights into the immunomodulatory roles of bovine HDPs, several limitations should be acknowledged. The i*n vitro* experiments using isolated PBMCs and BT cells may not fully reflect the complexity of immune responses in vivo. In addition, only a limited panel of cytokines was measured, and cytokine kinetics were not assessed. Future studies using whole blood, more animals, and a broader cytokine profiling would be needed to confirm these findings and provide a wider overview of the immunomodulatory effects of HDPs.

Conclusions

Our findings demonstrate that BNBD1, BNBD3, LAP, Bac5, and BMAP27 exhibit distinct immunomodulatory profiles on PBMCs and BT epithelial cells. Prophylactic treatment with BNBD1, BNBD3 and LAP significantly enhanced LPS-induced inflammatory responses, suggesting their potential as immune-priming agents. Notably, LAP also triggered IL-8 secretion in unstimulated PBMCs, indicating an intrinsic chemotactic capability. BMAP27 exhibited a clear immunosuppressive effect, suggesting a therapeutic potential in controlling excessive inflammatory responses such as those occurring in BRD. These results underscore the functional diversity of bovine HDPs, beyond their antimicrobial roles, and their potential for therapeutic and prophylactic applications in cattle.

Abbreviations

HDPs	Host defense peptides
BNBD1	Bovine neutrophil β-defensin 1
BNBD3	Bovine neutrophil β-defensin 3
Bac5	Bactenecin 5
BMAP27	Bovine myeloid antimicrobial peptide 27
LAP	Lingual antimicrobial peptide
TAP	Tracheal antimicrobial peptide
LPS	Lipopolysaccharides
BT	Bovine turbinate
PBMCs	Peripheral blood mononuclear cells
RTCA	Real-time cell analysis
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1α
TGF-β1	Transforming growth factor-β1
BRD	Bovine respiratory disease

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

Conceptualization, E.G.-F. and A.A; methodology, C.S. S.T.-A.; formal analysis, C.S; investigation, C.S. and S.T.-A.; resources, E.G.-F., A.A. and K.G.M; writing, C.S., E.G.-F., A.A. and K.G.M; supervision, E.G.-F., A.A. and K.G.M; funding acquisition, E.G.-F., A.A. and K.G.M. All authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study obtained ethical exemption for blood samples from the Animal Research Ethics Committee (AREC) at University College Dublin (AREC-E-22-34-Meade) and Animal Care Committee of the Generalitat de Catalunya (project number 12287).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Ruminant Production, IRTA, Torre Marimon, Caldes de Montbui, Catalonia 08140 , Spain
[2]UCD School of Agriculture and Food Science, University College Dublin, Belfield, Dublin V1W8, Ireland
[3]UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin D04 V1W8, Ireland
[4]UCD One Health Centre, University College Dublin, Belfield, Dublin D04 V1W8, Ireland
[5]UCD Institute of Food and Health, University College Dublin, Belfield, Dublin D04 V1W8, Ireland

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