RESEARCH ARTICLE

LL-37, HNP-1, and HBD2/3 modulate the secretion of cytokines TNF-α, IL-6, IFN-γ, IL-10 and MMP1 in human primary cell cultures

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ABSTRACT. The aim of this study was to evaluate the effects of the LL-37, HNP-1 and HBD2/3 peptides on cytokine and MMP production in human polymorphonuclear cells, mononuclear cells and chondrocytes. The levels of cytokines in supernatants from mononuclear and polymorphonuclear cell cultures were measured with a cytometric bead array by flow cytometry. Likewise, the levels of metalloproteinase/MMP-1, 3, and 13 were measured in supernatants from chondrocyte cultures using an ELISA. The expression of RANKL on lymphocytes was analyzed by flow cytometry. We observed increased levels of TNF-α, IL-6 and IL-10 in mononuclear and polymorphonuclear cell cultures stimulated with HBD-2/3. We also observed increased levels of IFN-γ, IL-10, and IL-6 in mononuclear cell cultures stimulated with HNP-1, and increased IL-6 levels were observed in polymorphonuclear cell cultures exposed to HNP-1. We also found that the MMP-1 level increased in the chondrocyte cultures stimulated with HBD-3, whereas the MMP-1 level was decreased in cultures exposed to LL-37. The present report is the first study to determine that HNP-1and HBD2/3 promote the secretion of pro-inflammatory cytokines by polymorphonuclear and mononuclear cells and the secretion of MMP by chondrocytes, whereas LL-37 diminishes MMP1 secretion. Our results suggest that HBD-2/3 and HNP1 might play a pathological role in rheumatoid arthritis, while LL-37 might have a protective role.

Key words: host defense peptides, inflammatory cytokines, matrix metalloproteinases, rheumatoid arthritis, chondrocyte culture, neutrophil culture

Human defense peptides (HDP) are small cationic and amphipathic molecules of variable length, sequence and structure that act as a natural defense mechanism. HDPs kill by permeabilizing the cytoplasmic membranes of pathogenic microorganisms [1]. It is important to note that HDPs may also modulate the responses of different subtypes of cells when they interact with a membrane receptor such as P2X7, FPRL1, the chemokine receptor CCR6/2, or the toll-like receptor (TLR)-1 and -2 [2-5]. In addition, HDPs have been implicated in autoimmune diseases such as rheumatoid arthritis (RA). The antimicrobial peptides human β-defensin (HBD)-3 and LL-37, a cathelicidin, have been detected in the synovial membrane of RA patients [6]. It was recently reported that increased levels of α-defensins (HNP) and BPI in synovial fluid and serum from patients with RA were associated with joint erosion and leucocyte count [7]. In addition, it has been reported that HDB-3 participates in the tissue remodeling process of articular cartilage; it also induces the production of matrix metalloproteinases (MMP) and reduces levels of tissue inhibitors of metalloproteinases (TIMP)-1 and -2 [8]. Furthermore, LL-37 reduces osteoclast cell number by promoting their apoptosis [9]. Increased expression of LL-37 in neutrophil granulocytes and macrophages/osteoclasts in the synovium during RA has been described [10], and the expression of LL-37 has been linked with increases in cell death, interferon (IFN)-α level, and the development of autoantibodies during pristane-induced arthritis [10].

In RA, mainly mononuclear and polymorphonuclear cells infiltrate synovial tissue. The activation of these cells results in the release of pro-inflammatory cytokines that contribute to joint inflammation. The production of MMP by chondrocytes is also a contributing factor in the degradation of articular cartilage in RA patients. Considering

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that some HDPs are present in high levels in synovial fluid of RA patients with articular damage and they might promote the expression and secretion of molecules involved in bone erosion and cartilage degradation during RA, the aim of this study was to determinate the effect of human defense peptides LL-37, HBD-2/3, HNP-1 on 1) secretion of matrix metalloproteinases (MMP)-1, 3 and 13 in primary chondrocyte cultures, 2) secretion of pro-inflammatory IL-2, IL-4, IL-6, IL-10, TNF-a, and IFN-γ in mononuclear and polymorphonuclear cell cultures, and 3) the expression of RANKL on lymphocytes.

METHODS

Chondrocyte culture

Human chondrocytes were isolated from a cartilage biopsy obtained during trauma surgery involving a 24-year-old patient. Chondrocytes were isolated as described [11], and cultured at 37°C, in a 5% CO_2 atmosphere and 100% humidity. Chondrocyte proliferation was evaluated by inverted microscopy, and cell cultures were not allowed to reach more than 80% confluence. Chondrocyte lineage was assessed by polymerase chain reaction analysis of the expression of the following genes: type I collagen, type II collagen and SOX-9. As expected, SOX9 and type II collagen genes were expressed continuously for at least 21 days [12]. Chondrocytes were frozen and kept at -80°C for approximately 90 days, until experimental evaluation. Subsequently, cells were thawed and 1×10^6 cells/mL were cultured in 25-cm^2 flasks with RPMI-1640, supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. To avoid phenotypic changes, cells were maintained in culture for up to four passages, which corresponded approximately to a period of 19 days; no changes in characteristic chondrocyte morphology were observed during this period of time. Afterward, cultures were harvested, and 2×10^5 cells/mL/well were seeded onto 24-well plates for overnight incubation as described above. Treatments were applied as follows: IL-1β (eBioscience, Inc., San Diego, CA, USA) (100 pg/mL, 100 ng/mL, 500 ng/mL), human α-defensin-1 (HNP-1) (Bio-Rad AbDSerotec Inc. Raleigh NC, USA) (10 ng/mL, 0.1 μg/mL, 1 μg/mL), human β defensin-2 (HBD-2) (Bio Basic Inc., Bailey Avenue, Amherst NY, USA) (10 ng/mL, 0.1 μg/mL, 1 μg/mL) and cathelicidin (LL-37) (Hycult BiotechInc. Plymouth Meeting, PA, USA) (10 ng/mL, 0.1 μg/mL, 1 μg/mL), for 24 h at 37°C in 5% CO_2. After incubation, supernatants were collected and stored at -70°C until use.

Peripheral blood mononuclear cells (PBMC) isolation and culture

PBMCs were isolated from peripheral blood samples, obtained from healthy subjects, by Ficoll-Hypaque (Sigma Chemical Co., St Louis, MO, USA) density-gradient-centrifugation as described previously [13]. Cells were then washed and re-suspended at 1×10^6 cells/mL in RPMI-1640 complete culture medium (Hyclone, Laboratories Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Rockville, MD, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). Cell viability was assessed using the Trypan blue exclusion test. Finally, the cells were stimulated with phytohemagglutinin (PHA) (20 g/mL), IL-1β (100 pg/mL, 100 ng/mL, 500 ng/mL), HNP-1 (1 μg/mL, 5 μg/mL, 10 μg/mL), HBD-2, (1 μg/mL, 5 μg/mL, 10 μg/mL) and LL-37 (10 ng/mL, 0.1 μg/mL, 1 μg/mL) Santa Cruz Biotechnology, Dallas, Texas, USA) or medium alone for 18 hours at 37°C, and in 5% CO_2. Finally, supernatants were collected and stored at -20°C until analysis.

Polymorphonuclear (PMN) leucocyte isolation and culture

Peripheral blood samples obtained from healthy subjects were placed in tubes containing 500 μl of the anticoagulant citrate dextrose solution (ACD). Dextran sedimentation was performed using 3 mL of a 6% dextran solution. The samples were then incubated at 4°C, in the dark. The layer containing the leukocytes was extracted and deposited in a tube, and then the remaining erythrocytes were eliminated by hypotonic lysis. The cell suspension was then layered over a Ficoll-Paque density gradient (Sigma, San Diego CA, U.S.A) for purification of the PMN cells. The pellet corresponding to the PMN cells was then washed, and cell viability was assessed using the Trypan blue exclusion test. The neutrophil composition in this fraction was evaluated by flow cytometry using a monoclonal antibody against human CD66b (eBiosciences, Inc. San Diego CA USA) conjugated with allophicocianin (APC). We observed that over 90% of cells tested positive for CD66b. Subsequently, 2×10^5 PMNs were stimulated with phytohemagglutinin (PHA) (20 μg/mL); lipopolysaccharide (LPS) (10 μg/mL) (Sigma-Aldrich); LL-37 (5 μg/mL), HNP-1 (5 μg/mL); HBD-2 (5 μg/mL) and HBD-3 (5 μg/mL, 10 μg/mL) (Santa Cruz Biotechnology Dallas, Texas USA). The cells were then incubated for 18 hours in RPMI 1640 in a complete culture medium at 37°C and at 95% humidity. Finally, the supernatants were collected and stored at -70°C until further analysis.

Matrix metalloproteinases measurement

The matrix metalloproteinase levels in the chondrocyte culture supernatant were measured by ELISA. Briefly, aliquots of the chondrocyte culture supernatant were either stimulated or not stimulated, and were assayed for the presence of MMP-1, MMP-3 and MMP-13 using three commercial kits (eBiosciences, Inc. San Diego, CA, USA). These detected active and non-active MMP. The signal was detected by a spectrophotometer (Multiskan Ascent Plate Reader, MTX Lab Systems, Inc.) at 466 nm for three independent experiments performed in duplicate.

Interleukin measurements

In order to measure the levels of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor (TNF) -α, and interferon-γ (IFN-γ) protein in culture supernatants, a cytometric bead array (CBA, BD Biosciences, San Jose, CA, USA) was performed in accordance with the manufacturer's recommendations. Data acquisition and analysis was

then performed using a FACS Canto II Flow Cytometer equipped with DIVA software (DB Biosciences, San Jose, CA, USA).

Flow cytometry analysis

PBMCs were stimulated with HBD-2/3, HNP-1 and LL-37 as described above, then immunostained using a human TRANCE/RANKL allophycocyanin monoclonal antibody (R&D systems, Minneapolis, MN, USA) for 30 minutes at 4°C, in the dark. The cells were washed and fixed with 1% paraformaldehyde, and then analyzed using Diva software and a FACS Canto II flow cytometer (DB Biosciences, San Jose, CA, USA).

Statistical analysis

For statistical analysis, the Graph Pad Prism 5.0 software was used (San Diego, Calif. USA), and to determine the normality of the data, the Shapiro Wilk test was performed, and lastly for the evaluation to determine the differences between the means, a one-way analysis of variance (ANOVA) with a *post hoc* Tukey test was performed.

Ethics

Approval to perform the venipunctures was given by the institutional review board at the Mexican Institute for Social Security (IMSS). Written and informed consent was obtained from all study participants in accordance with the guidelines of the National Committee of Ethics at IMSS and in accordance to with Helsinki's declaration.

RESULTS

To gain insight into the role of HDPs in the secretion of cytokines that contribute to the perpetuation of inflammation in RA joints, we decided to evaluate the effect of HNP-1, LL-37, and HBD-2/3 on cytokine production. To this end, PBMC and PMN cultures were used. First, as a control, using PBMC cultures, we measured the secretion of TNF-α, IFN-γ, IL-10, IL-6, IL-4 and IL-2 in response to a polyclonal stimulus PHA (*figures 1A-F*). Then, as can be seen (*figures 1B-D*), HNP-1 induced the secretion of IFN-γ, IL-10, and IL-6 in PBMC cultures. Also, HBD-3 triggered the secretion of TNF-α, IL-10, and IL-6 by PBMC cultures (*figures 1A, C, D*). However, neither LL-37 nor HBD2 had any effect on the secretion of cytokines in PBMC cultures (*figures 1A-F*). We next evaluated whether HDPs induced the secretion of cytokines in PMN cultures. We found that under our culture conditions, which comprised >90% neutrophil, only the secretion of IL-6 was induced by HNP-1 (*figure 2D*). Likewise, HBD2/3 induced secretion of IL-6, as well as TNF-α and IL-10 (*figures 2A, C, D*). To control for effectiveness of the cytokine assays, the effect of a polyclonal stimulus with LPS on PMN cultures was measured. As expected, LPS induced the secretion of TNF-α, IL-10, and IL-6 (*figures 2A, C, D*), but not of IFN-γ, IL-4 or IL-2 (*figures 2B, E, F*).

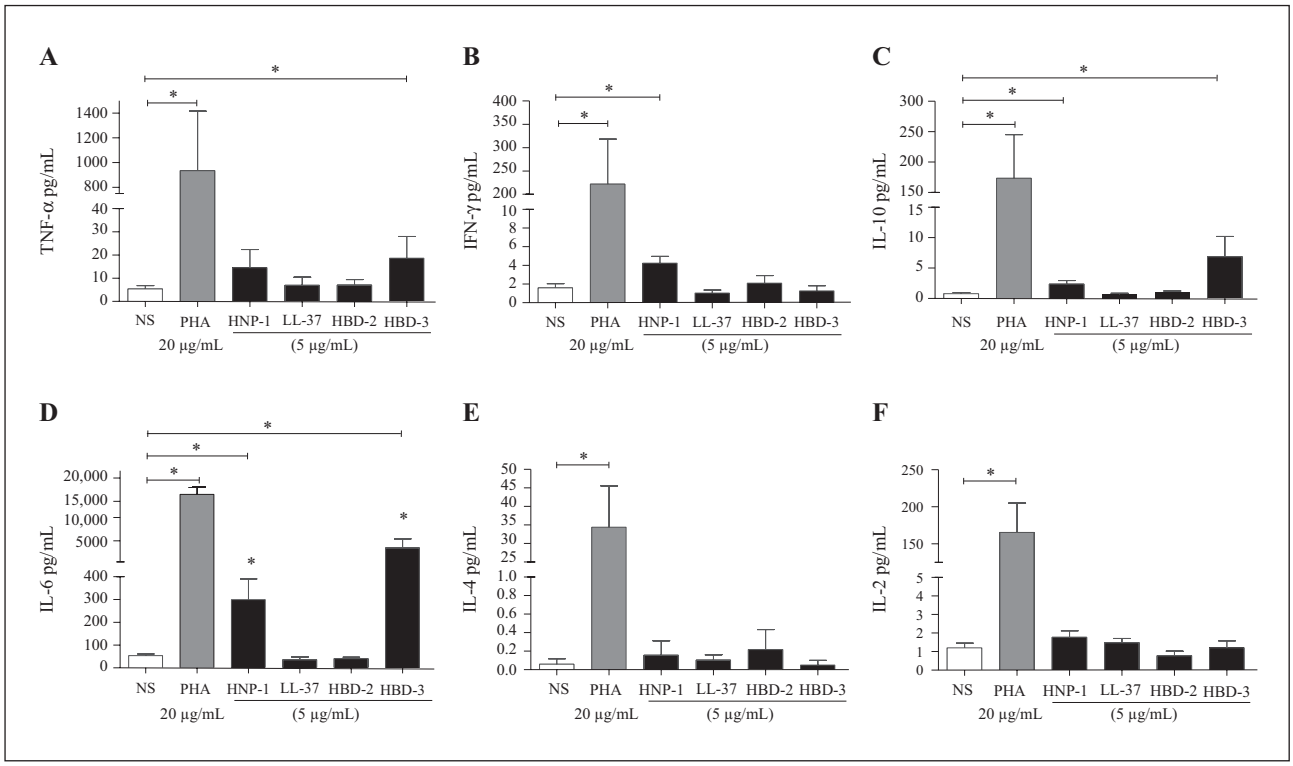

Figure 1

Levels of TNF-α, IFN-γ, IL-10, IL-6, IL-4, IL-2 in supernatants of mononuclear cell cultures stimulated with HBD-2/3, HNP-1, and LL-37. PBMCs were purified from a heparinized blood sample and were then cultured under the conditions described in the methods and materials section. The protein levels of **(A)** TNF- α (n = 6), **(B)** IFN-γ (n = 6), **(C)** IL-10 (n = 6), **(D)** IL-6 (n = 6), **(E)** IL-4 (n = 6), **(F)** IL-2 (n = 6), were quantified in the culture supernatants using a cytometric bead array (CBA). NS = non-stimulated. The data were then assessed using six independent experiments for each culture condition. In the graphics, we show mean ± SD. The differences between the means were determined using a one-way analysis of variance (ANOVA) with a *post hoc* Tukey test, and a $p<0.5$ was considered significant. * Statistically significant differences compared to non-simulated condition.

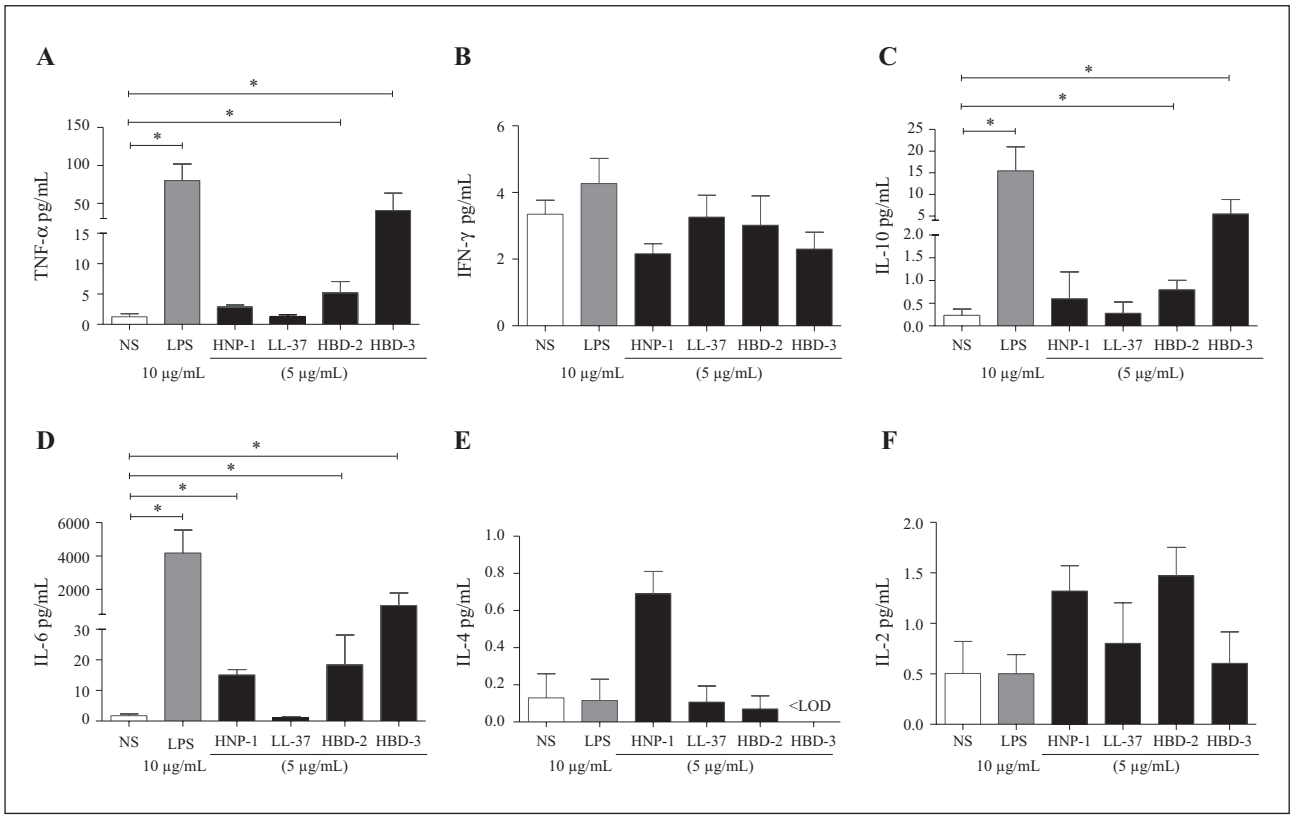

Figure 2

Levels of TNF-α, IFN-γ, IL-10, IL-6, IL-4, IL-2 in supernatants of polymorphonuclear cells (<90% neutrophils) cultures stimulated with HBD-2/3, HNP-1, and LL-37. Polymorphonuclear cells were purified from blood samples and were then cultured under the conditions described in our method and materials section. The percentage of CD66b-positive cells was then evaluated using a monoclonal antibody against CD66b antigen by flow cytometry: >90% of polymorphonuclear leukocytes were CD66-positive (neutrophils) (data not shown). The protein levels of **(A)** TNF-α (n = 4), **(B)** IFN-γ (n = 4), **(C)** IL-10 (n = 4), **(D)** IL-6 (n = 4), **(E)** IL-4 (n = 4) **(F)** IL-2 (n = 4), were quantified in the culture supernatants using a cytometric bead array (CBA). The data were assessed from four independent experiments for each culture condition. The graphics show that the mean ± SD: <LOD: results that fall below the detection limit. The differences between the means were determined using one-way analysis of variance (ANOVA) with a *post hoc* Tukey test: s a $p<0.5$ was considered a significant result. * Statistically significant differences compared to non-stimulated condition.

Recent work has suggested the implication of some HDPs in RA, and also in the induction of the expression of metalloproteinases (MMP). Therefore, using chondrocyte cultures, we tested the ability of HDPs to induce the secretion of MMP-1, 3, and 13. First, we found that, in comparison to non-stimulated cultures, IL-1β efficiently increased ($p<0.001$) the levels of MMP-1, 3 and 13 in cultures stimulated with different concentrations (*figures 3A-C*). When the effect of 1 µg/mL of HBD-2 was evaluated, an increase in the levels of MMP-1 and MMP-3 was observed. However only the increase in MMP-1 was statistically significant when compared to the control (*figures 3A, B*). Surprisingly, a decrease in the levels of MMP-1 in cultures of chondrocytes stimulated with 0.1 µg/mL and 1 µg/mL of LL-37 was found (*figure 3A*). Nevertheless, non-significant increases in the levels of MMP-1 and MMP-3 were found when chondrocyte cultures were stimulated with HNP-1 (*figures 1A, B*)

Finally, we evaluated whether HDPs could modulate the expression of the RANK ligand (RANKL) in PBMC lymphocytes. As can be seen (*figure 3D*), we detected an increase in the proportion of RANKL positive cells in cultures stimulated with PHA as a polyclonal stimulus: however, when PBMC cultures were treated with HNP-1 or LL-37, a small increase in the proportion of RANKL positive cells could be noticed, but it was not statistically significant in comparison to the non-stimulated cultures (*figure 3D*).

DISCUSSION

The joint inflammation seen in RA, is initiated and perpetuated by cytokines, which are produce by the different types of immune cells recruited into the synovium. In this study, we evaluated the effect of HDP on the secretion of cytokines in primary cultures of mononuclear cells and neutrophils. Our results show that HDPs such as HNP-1 and HBD2/3 induced the secretion of cytokines related to the pathophysiology of RA, such as TNF-α, IL-6 and IFN-γ. Our results are consistent with previous findings describing the capacity of HDPs to regulate the expression of IL-6 and IL-8 by fibroblast-like synoviocytes (by HNP-1) [14], or to modulate the secretion of IL-17A and IL-22 in epithelial cells (by HBD-3) [15]. Our results are also in line with previous results in which HNP-1 and HBD-1 enhanced the production of TNF-α, IL-6 and IL-12 in dendritic cells [16]. The induction of secretion of pro-inflammatory cytokines by HDPs might be implicated in the cytokine imbalance involved in the synovium destruction seen in RA [17]. Our findings that the secretion of

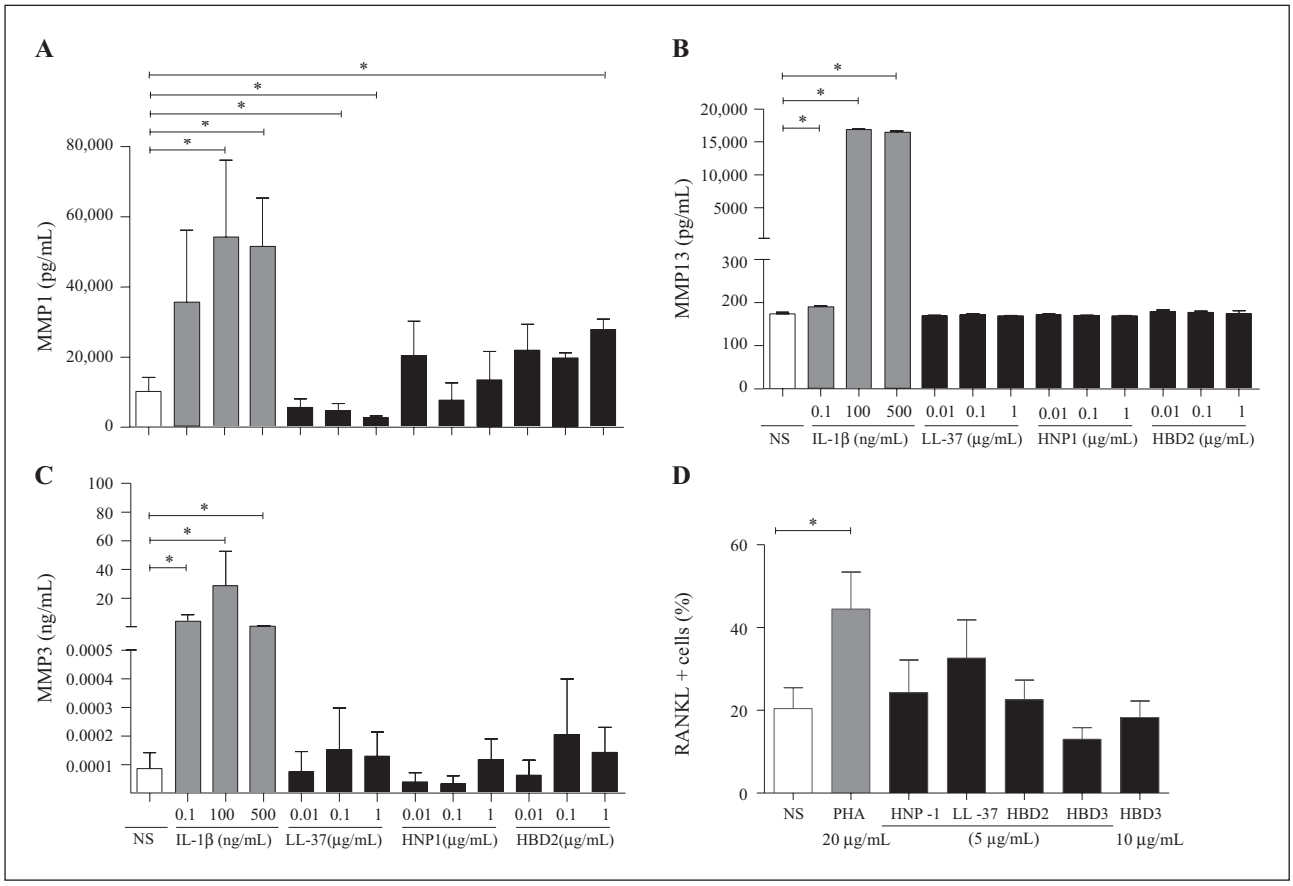

Figure 3

Levels of metalloproteinase/MMP-1, 3 and 13 in supernatants of chondrocyte cultures stimulated with different concentrations HBD-2/3, HNP-1, and LL-37. Human recombinant IL-1β was used as a positive control. The levels of **(A)** MMP-1 (n = 6), **(B)** MMP-13 (n = 6) and **(C)** MMP-3 (n = 6) were then measured in the chondrocyte culture supernatant using an ELISA. NS = non-stimulated. From these data we were able to assess three independent experiments, which were performed in duplicate. In order to evaluate the percentage of RANKL-positive cells, the PBMC were then stimulated with HNP-1, LL-37 and HBD-2/3 as described in our methods and material section. Then the PBMC were immunostained with a monoclonal antibody used against RANKL in conjunction with allophycocyanin (APC), which allows us to use, acquire and analyze in a flow cytometer. **(D)** The percentage of RANKL-positive cells was gated on a lymphocyte population (n = 6). The data were then assessed using six independent experiments. The graph shows the mean ± SD. Differences between the means were determined using a one-way analysis of variance (ANOVA) with a *post hoc* Tukey test: $p<0.5$ was considered a significant result.

IL-10 is induced by the antimicrobial peptides HNP-1 and HBD-3 in mononuclear cell cultures and by HBD2/3 in PMN cultures are in apparent contradiction with the studies performed in human dendritic cell cultures [16]. In those experiments, no effects of HNP-1 or HBD-1 on the production of IL-10 were found. IL-10 is a cytokine with inhibitory contributions to immune responses. In this regard, serum from RA patients has shown higher levels of IL-10 compared to controls, which seems to downregulate the expression of IL-6 [18]. However, another study has also shown that the synthesis of CCL8 by neutrophils requires the expression of IL-10. CCL8 is a chemokine critical for the recruitment of leukocytes into the synovial compartment; therefore, the induction of IL-10 by HNP-1 and HBD-3 could contribute to the pathogenesis of RA. Additionally, we hypothesize that the increase in IL-10 levels, in both PBMC and PMN cultures stimulated with HBD-3 and HNP-1, might be the consequence of induction of pro-inflammatory cytokines by these HDPs since it has been shown that induction of pro-inflammatory cytokines themselves may trigger the synthesis of IL-10 as a mechanism by which the cytokine network regulates inflammation [19]. However, this needs to be confirmed. Taken together, our results indicate that HDPs might have a role in the pathogenesis of RA through the induction of pro-inflammatory cytokines.

Joint destruction in RA is an event wherein proteolytic enzymes, such as matrix metalloproteinase (MMP) and osteoclast resorption, play a critical role [20]. In this study, we evaluated the effect of HBD-3, HBD-2, HNP-1 and LL-37 on the secretion of MMP-1, 3 and 13 using primary chondrocyte cultures. Whereas, we found increased levels of MMP-1 in chondrocyte cultures upon stimulation with HBD-2, previous reports have already described increased levels of MMP1 in supernatants of chondrocyte cultures exposed to HBD-3 [8]. They share substantial sequence identity and structural similarity. Therefore, our results suggest that HBD-2 and HBD-3 have a similar effect on MMP-1 secretion in human chondrocyte cultures, which is comparable to the basis of their antimicrobial activity against some pathogens [21]. In spite of other studies that have reported that LL-37 induces the expression of MMP-9 in fetal membranes [22], here we found a decrease in MMP-1 levels upon LL-37 stimulation. This discrepancy can be explained by the fact that both anti-inflammatory and pro-inflammatory properties have been attributed to LL-37 [23-26]. Despite this dual activity of LL-37, there are studies showing synergistic, anti-inflammatory effects

between LL-37 and HBD3 [27]. The relevance of MMP-1 in RA is actually well-described: whereas increased levels of MMP-1 have already been observed in synovial fluid as well as in the serum of RA patients, the increase MMP-1 correlates to the presence of joint erosions [28, 29]. Conversely, the inhibition of MMP-1 is suggested to have a chondroprotective effect [30]. In this regard, under our culture conditions we observed that HBD-2 increased the secretion of MMP-1; whilst LL-37 caused its decrease. Thus it is very likely that HBD-2 and LL-37 have contrasting roles in the degradation of the extracellular matrix.

In the scenario of RA, HDP secretion may occur after the activation of phagocytic cells, epithelial and chondrocytic cells by damage associated with molecular patterns (DAMPs), which are expressed in arthritic joints [31, 32]. Also, the secretion of HDPs may be induced by pro-inflammatory cytokines present in RA, such as TNF-α and IL-1β [33, 34]. According to our results, the presence of HDPs in RA joints may promote several pathogenic events such as the recruitment of dendritic cells [2], monocytes, T lymphocytes and neutrophils [34] from the periphery to the inflamed joint, and in turn induce the inflammatory process and cartilage degradation, promoting pro-inflammatory cytokine and MMPs secretion by infiltrated and resident cells. In this regard, it has been described that HDPs exert their effects by either direct binding or by transactivating several extracellular or intracellular receptors [35]. Thus, LL-37 can modulate inflammatory responses through a direct interaction with FPR-like 1 receptor (FPRL1) [36] and the P2X7 purinergic receptor [37]. In addition, LL-37 may downregulate TLR-mediated pro-inflammatory signals by different mechanisms, including the inhibition of NF-kB subunit translocation [38] and the inhibition of interaction of lipopolysaccharide (LPS) with LPS-binding protein (LPB) and MD2 [39]. Likewise, it has been described that HBD2 and 3 may affect the pro-inflammatory signaling pathways induced through the immune innate receptors TLR1, TLR2, TLR9 and CCR2 [4, 40].

In regards to the effect of HDPs on RANKL expression we observed that no change was seen in the proportions of RANKL positive cells that were stimulated with HNP-1, LL-37, HBD-2 and HBD-3. The mechanism by which the HDPs are associated with the presence of bone erosions may consist only of their capacity to increase the secretion of IL-6 and TNF-α, thus causing the inflammatory cytokines to participate in the bone destruction and swollen joints seen in RA [41, 42].

Our findings indicate that HBD-2/3 and HNP-1 might play a role in RA by promoting the secretion of pro-inflammatory cytokines and MMP, whereas LL-37 might play a protective role by preventing extracellular matrix degradation through diminishing MMP-1 secretion. In this regard, it worth mentioning that HDPs are synthesized by different cell types, which can modulate distinct phenomena. In addition, HDP effects may differ in the presence of pro-inflammatory stimuli such as cytokines and LPS [43]. Therefore, it would be of interest to further assess the possible role of HDP in the pathogenesis of inflammatory autoimmune conditions, taking into account their possible synergic or antagonistic effects with other mediators, mainly pro-inflammatory cytokines.

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